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Role of lipid-modifying enzymes in oat and faba bean: synthesis of off-flavour compounds

Zhen Yang

ACADEMIC DISSERTATION

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ABSTRACT

The increasing use of plant-derived foods, especially their proteins, is a global trend. However, when oat and faba bean are used as food materials, lipid deterioration may occur and result in undesirable off-flavours. Lipid-modifying enzymes, together with chemical reactions, are the main factors responsible for the formation of off-flavour compounds in plant-derived foods. However, little is known about the overall effects of these lipid-modifying enzymes on the level of and variations in enzyme activities in oat and faba bean seeds and their products in terms of off-flavours. Understanding and controlling the formation of lipid-related off-flavours in oat and faba bean is crucial to prolong shelf life and to earn consumers' acceptance.

The aim of this thesis was to study the role of lipid-modifying enzymes in oat and faba bean. To reach this aim, the levels of and variations in the lipid-modifying enzyme activities present in oat and faba bean seeds from selected cultivars and cultivation years were studied (Study I). In addition, the occurrence and formation of non-volatile oxidised fatty acids (NVOFAs) by lipid-modifying enzymes in oat were investigated (Study II). Finally, the role of lipid-modifying enzymes in the formation of off-flavour compounds in faba bean foods was studied (Study III). Furthermore, analytical methods were developed to study peroxygenase (POX) activity and analyse lipid-derived NVOFAs occurring in oat.

POX activity was measured based on the production of epoxy compounds from substrates with cumene hydroperoxide added as an oxidant. The epoxy products formed were measured using two reliable and sensitive methods: gas chromatography coupled with flame ionisation detection or mass spectrometry (GC-FID/MS) and ultra-high performance liquid chromatography coupled with evaporative light scattering detection or MS (UHPLC-ELSD/MS). In addition, the accelerated solvent extraction (ASE) method was used to extract the total lipids from oat with high yields, and the solid phase extraction (SPE) method showed good purification and recovery for NVOFAs. Finally, the developed UHPLC-ELSD/MS method could reliably analyse the NVOFA products in oat.

Lipase and POX activities were found in oat grains, but lipoxygenase (LOX) activity was not. The lipase activity may be affected by both the oat cultivars and cultivation years as well as the interactions between the two, but the POX activity was affected only by cultivars. Oat POX preferred FFAs to their methyl esters, and the enzyme catalysed substrates at various levels of unsaturation to form mono-epoxy products. Triacylglycerols (TAGs) were not substrates for oat POX. Because of POX activity in oat, NVOFAs occurred in the flours of non-heat treated (NHT) oat grains, and their amounts increased remarkably during the storage of oat samples. The formation of NVOFAs was in line with the release of FFAs by oat lipase. In addition, the formation of NVOFAs in the flour of heat-treated (HT) oat grains was quite small.

Faba bean possessed high levels of lipase and LOX activities, but no POX activity. Both faba bean lipase and LOX activities were significantly affected by cultivars, but only LOX activity was also affected by cultivation years. Faba bean lipase possessed optimum pH at 8 using *p*-nitrophenyl butyrate (*p*-NPB) as a substrate. However, the lipase hydrolysed more TAG substrates at pH 7.5 than at pH 8, with the order being triolein > trilinolein > rapeseed oil. Faba bean LOX had optimum pH at 6 using linoleic acid as a substrate, and it produced a majority of 9-hydroperoxyoctadeca-10(*E*),12(*Z*)-dienoic acid (*ca.* 60% of total hydroperoxides). The LOX pathway produced much more volatile lipid oxidation products from linoleic and linolenic acids than from TAGs. In addition, linoleic and linolenic acids produced specific profiles of volatile compounds. Adding rapeseed oil in emulsions increased the formation of volatile lipid oxidation products, and adding rapeseed oil fatty acids increased it even more. Our studies showed that the pH level greatly affected the extent of the reactions. The highest amounts of volatiles were formed at pH 6.4, at which both lipase and LOX may possess activities.

Overall, this thesis evaluated the role of reactions catalysed by lipid-modifying enzymes together with chemical lipid oxidation for their potential to form lipid-derived off-flavours in oat and faba bean. Reliable methods were developed for analysing POX activity and NVOFAs in oat. Heat treatment was proven to effectively inhibit formation of NVOFAs and degradation of lipids during oat storage. Finally, when faba bean is used as a food ingredient, the inactivation of lipase and LOX activities is crucial to maintaining stable and high-quality faba bean food products.

PREFACE

This thesis was carried out during the years 2014-2019 in the Food Chemistry Group, Department of Food and Nutrition (previously known as Department of Food and Environmental Sciences), Faculty of Agriculture and Forestry, University of Helsinki. Financial supports come from the China Scholarship Council, Finnish Cultural Foundation, Finnish Food Research Foundation (ETL) and Dissertation Completion Grant at the University of Helsinki. All funding supporters are greatly appreciated and acknowledged.

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Last but not least, I give my deepest thanks to my parents for their unconditionally supports and cares throughout these years!

Zhen Yang
Helsinki, November 2019

"SUCCESS IS NOT FINAL, FAILURE IS NOT FATAL.
IT IS THE COURAGE TO CONTINUE THAT COUNTS."
-WINSTON CHURCHILL-

Contents

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications and manuscript, which are referred to by Roman numerals I–III in the text.

I. Yang Z., Piironen V., Lampi A.-M. 2017. Lipid-modifying enzymes in oat and faba bean. *Food Res Int*. 100:335–343.

II. Yang Z., Piironen V., Lampi A.-M. 2019. Epoxy and hydroxy fatty acids as non-volatile lipid oxidation products in oat. *Food Chem*. 295:82–93.

III. Lampi A.-M., Yang Z., Mustonen O., Piironen V. 2019. Potential of faba bean lipase and lipoxygenase to promote formation of volatile lipid oxidation products in food models. *Submitted*.

Contribution of the author to papers I–III:

I. Zhen Yang planned the studies with the other authors and conducted the experimental works. He had the main responsibility for interpreting the results and for preparing the manuscript. He acted as the corresponding author of the paper.

II. Zhen Yang planned the studies with the other authors and conducted the experimental works. He had the main responsibility for interpreting the results and for preparing the manuscript. He acted as the corresponding author of the paper.

III. Zhen Yang planned the study with the other authors and conducted most of the experimental work. The experiment was partly conducted as a Master's Thesis (O. M.), and Zhen Yang was co-responsible for supervising this experimental work. Zhen Yang was involved in interpreting the results and preparing the manuscript. He acted as the corresponding author of the paper.

ABBREVIATIONS

ADH	Alcohol dehydrogenase
ASE	Accelerated solvent extraction
Asp	Aspartate
DAG(s)	Diacylglycerol(s)
DCM	Dichloromethane
DVB/CAR/PDMS	Divinylbenzene-carboxen-polydimethylsiloxane
ESI	Electrospray ionisation
FFAs	Free fatty acids
GC-FID/MS	Gas chromatography coupled with flame ionisation detection or mass spectrometry
His	Histidine
13-HOT	13-hydroxyoctadeca-9(*Z*),11(*E*),15(*Z*)-trienoic acid
HPL	Hydroperoxide lyase
9-HPOD	9-hydroperoxyoctadeca-10(*E*),12(*Z*)-dienoic acid
13-HPOD	13-hydroperoxyoctadeca-9(*Z*),11(*E*)-dienoic acid
13-HPOT	13-hydroperoxyoctadeca-9(*Z*),11(*E*),15(*Z*)-trienoic acid
HS-SPME-GC-MS	Headspace solid-phase microextraction coupled with gas chromatography and mass spectrometry
HT	Heat-treated
LC	Lipid chromatography
LOX	Lipoxygenase
MAG(s)	Monoacylglycerol(s)
NHT	Non-heat-treated
NP-HPLC	Normal phase high performance liquid chromatography
NVOFAs	Non-volatile oxidised fatty acids
POX	Peroxygenase
p-NPB	*p*-nitrophenyl butyrate
p-NPP	*p*-nitrophenyl palmitate
PUFAs	Polyunsaturated fatty acids
Q-TOF	Quadrupole-time-of-flight
RO	Rapeseed oil
ROFA	Rapeseed oil fatty acids

RP-HPLC	Reversed phase high performance liquid chromatography
Ser	Serine
SPE	Solid phase extraction
TAG(s)	Triacylglycerol(s)
TLC	Thin-layer chromatography
UHPLC-ELSD/MS	Ultra-high performance liquid chromatography coupled with an evaporative light scattering detection or mass spectrometry

1 INTRODUCTION

Oat (*Avena sativa*) and faba bean (*Vicia faba* L.) are good protein sources, and their increased use would improve self-sufficiency in plant proteins and contribute to the sustainable development of agricultural and food systems in the EU (Bues et al. 2013). Oat is a valuable source of nutrients, including proteins, unsaturated fatty acids (UFAs), soluble dietary fibre (β-glucan), vitamins and minerals, for both human beings and animals (Decker et al. 2014). The health benefits of β-glucan in oat have been intensively studied (Daou and Zhang 2012; Decker et al. 2014). Faba bean (*Vicia faba*), also known as fava bean, or broad bean, belongs to the Fabaceae family and is one of the leading grain legumes in the world (Lim 2012). It is a rich source of proteins (Khan et al. 2015; Lizarazo et al. 2015). Large faba beans (*V. faba major*) are used as food ingredients and a source of proteins in many countries and areas in the world, such as South China, India, the Middle East and North Africa (Crepon et al. 2010; Jensen et al. 2010), while small-grain faba beans (*V. faba minor*) are cool-season crops cultivated in many regions and consumed as food and feed (Crepon et al. 2010; Link et al. 2010).

When plant materials are processed into food products, oxidative deterioration of lipids may result in volatile off-flavour compounds (causing e.g. 'beany' off-flavour), or non-volatile off-flavour compounds (causing e.g. bitter taste). These reactions are also risks in oat and faba bean. Oat has a high content of unsaturated lipids and is known to possess lipase activity (Lehtinen and Kaukovirta-Norja 2011). Faba bean also contains lipid-degrading enzymes, including lipoxygenase (LOX) (Chang and McCurdy 1985). Thus, in both oat and faba bean, lipid deterioration can occur as a result of a number of complex enzymatic and chemical reactions. This deterioration may cause decreased sensory quality due to synthetised undesired flavour compounds, decreased nutritional value and even compounds that have possible adverse health effects. Lipid-modifying enzymes catalyse the reactions in plant materials very quickly and efficiently. Oat (Lehtinen and Kaukovirta-Norja 2011) and faba bean (Crepon et al. 2010) are among the plant materials in which the role of enzymatic deterioration has been shown to be especially important. Thus, it is crucial to control these undesired reactions in order to maintain the acceptable sensory quality of oat and faba bean products.

Lipid deterioration often begins with the action of a lipid-hydrolysing enzyme, namely lipase, which liberates free fatty acids (FFAs) from their esters. These liberated fatty acids include polyunsaturated linoleic and linolenic acids, which can act as substrates for the lipid-oxidising enzymes (Lehtinen et al. 2003; Doehlert et al. 2010). Meanwhile, chemical oxidation of the

FFAs may also occur. Lipase activity and its effects on oat have been well characterised in previous studies (Lehtinen et al. 2003; Decker et al. 2014). Usually, some processing methods are required to inactivate lipase activity in oat to avoid flavour problems (Lehtinen et al. 2003). Lipase has also been isolated from small faba bean (Dundas et al. 1978), and a sequential hydrolytic pathway has been proposed (Henderson et al. 1981). However, little is yet known of lipase activity and its effects in faba bean.

Oxidation of fatty acids may be initiated by autoxidation, photo-oxidation or LOX, all of which lead to hydroperoxides as primary products. Hydroperoxides can further react to form volatile off-flavour products (e.g. hexanal) or non-volatile compounds (e.g. oxoacids) through several chemical and enzymatic pathways (Gardner 2003). Some compounds develop immediately through the action of enzymes, and some occur only after long-term storage.

LOX has been regarded as responsible for the development of off-flavours in legumes. It catalyses oxidation of free polyunsaturated fatty acids (PUFAs) into hydroperoxides, which can decompose further and provide the undesirable 'beany' flavour caused mainly by volatile compounds (Roland et al. 2017). Two LOX isoenzymes were characterised from faba bean (Clemente et al. 2000), and faba bean was found to possess medium-level LOX activity for a legume (Chang and McCurdy 1985). Little research has been done on LOX activity in cereals such as oat.

Peroxygenase (POX) is an enzyme that can potentially produce off-flavours due to its ability to catalyse the hydroperoxide-dependent conversion of UFAs into non-volatile products, such as epoxy and hydroxy fatty acids (Hamberg and Hamberg 1996), which have been suggested to be associated with bitter taste (Biermann et al. 1980; Doehlert et al. 2010). Hydroxy and epoxy fatty acids are the final products of multi-step oxidation processes. They have been reported in oat (Doehlert et al. 2010). POX has been isolated from oat grains (Hamberg and Hamberg 1996). Oat POX has been used to produce fatty acid epoxides in the chemical industry (Piazza et al. 2003c). Hamberg and Fahlstadius (1992) also reported the hydroperoxide-dependent epoxidation of UFAs in faba bean. However, little is known about POX activity in oat and faba bean foods.

The overall aim of this thesis was to study the role of lipid-modifying enzymes in oat and faba bean. The literature review mainly summarised the lipid degradation that causes off-flavour

products, with a main focus on enzymatic reactions. In addition, the thesis introduced methods to analyse the products related to off-flavour lipid degradation in cereals and legumes. The experimental part of the thesis included three studies: studying the levels of and variations in lipid-modifying enzyme activities present in oat and faba bean from selected cultivars and cultivation years (Study I), investigating the occurrence and formation of non-volatile lipid oxidation products by lipid-modifying enzymes in oat (Study II) and studying the role of lipid-modifying enzymes in the formation of off-flavour compounds in faba bean foods (Study III). Furthermore, in this thesis, analytical methods were developed to study POX activity and analyse lipid-derived non-volatile off-flavour compounds in oat.

2 LITERATURE REVIEW

2.1 Lipid degradation as a cause of off-flavour products

The degradation of lipids can lead to undesired flavours, texture changes and even compounds that have possible adverse effects on health (Ho and Chen 1994; Bartosz and Kolakowska 2011). The mechanism of lipid degradation and the formation of lipid-derived rancidity has been studied extensively in various food materials (Frankel 1984; Ho and Chen 1994; Feussner et al. 2001; Doehlert et al. 2010; Bartosz and Kolakowska 2011; Ties and Barringer 2012; Lampi et al. 2015). Even so, all the degradation pathways and factors that may affect the quality of foods are not completely clear. Thus, it remains an ongoing challenge to control lipid degradation in complex food systems.

2.1.1 Chemical lipid oxidation

2.1.1.1 Autoxidation

Autoxidation mechanism

Autoxidation is a reaction mediated by free radicals that involves triplet oxygen ($^3O_2$) and unsaturated lipids and that generally involves three phases: chain initiation, propagation and termination (Frankel 1984). A classical scheme of lipid oxidation by free-radical reactions was given by Schaich et al. (2013) and is shown in Fig. 1.

In the initiation stage, alkyl radicals are primarily formed by removing a hydrogen atom from a lipid (Ho and Chen 1994). The abstraction of hydrogen and the formation of alkyl radicals are catalysed by initiators, such as trace metals, UV light, heat energy and radiation (Frankel 1984).

In the propagation stage, the lipid free radical (L•) is active and reacts easily with molecular $^3O_2$, yielding a peroxyl radical (LOO•), which is also quite reactive and subsequently splits off a hydrogen atom from another unsaturated fatty acid, resulting in a hydroperoxide (LOOH) and another free radical (L•). Since the reaction of the free radical with $^3O_2$ is faster than that of peroxyl radicals with fatty acid hydrocarbon chains, the latter reaction determines the reaction rate of the autoxidation. The dissociation energy of allylic hydrogens, especially from a bisallylic position, is much lower than from other carbon positions. Thus, the autoxidation rate increases with the unsaturation level of the lipids, because more allylic carbon positions are available (Schaich et al. 2013). For instance, linolenate oxidises 20–30 times faster than linoleate, which oxidises 10 times faster than oleate (Ho and Chen 1994). The hydroperoxides formed are unstable and can decompose which lead to chain branching (Choe and Min 2006;

Schaich et al. 2013). Reactions catalysed by metal are heterolytic in hydroperoxide decompositions, yielding one radical and one OH^- or one H^+, as shown in the chain branching stage in Fig. 1 (Schaich et al. 2013), while heat- and UV-induced homolytic scissions can produce both alkoxyl (LO•) and hydroxyl (HO•) radicals that react faster than LOO• and also may attack more lipid sites. Some radicals formed from scissions can rearrange internally into non-radical secondary products, such as aldehydes, while most may react further with oxygen and produce peroxyl radicals, which can abstract hydrogen from another lipid (LH) and finally form a new hydroperoxide and L•.

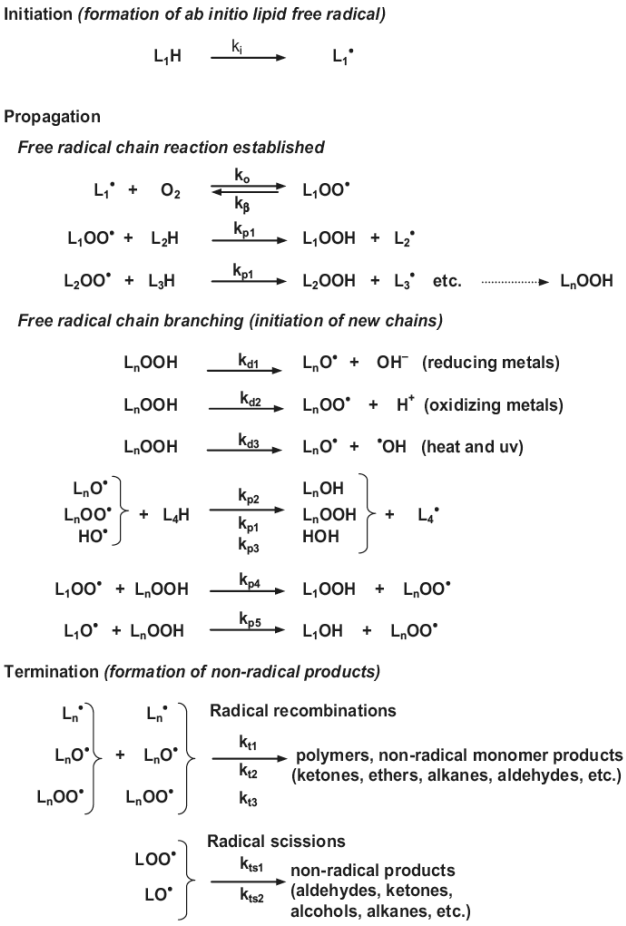


Fig. 1. A classic mechanism for free radical chain reaction for lipid oxidation (Adapted from Schaich et al. (2013), with permission to use).

The termination stage is the final phase of lipid autoxidation, in which the free radicals are terminated and form non-radical products through various mechanisms. There are four major pathways: radical re-combinations, α and β scissions of alkoxyl radicals, co-oxidation with other non-lipid molecules (e.g. proteins) and group eliminations (Schaich et al. 2013). A wide range of oxidation products is formed by radical re-combinations, which are usually responsible for the characteristics of oxidised lipids, with the most important products including mainly alkane polymers, alcohols and ketones, alkyl peroxides, ethers and peroxides. Co-oxidation is a process in which the lipid free radicals are terminated by non-lipid molecules. For example, the lipid radicals may react with proteins. Compared with the abovementioned three termination mechanisms, group elimination is less important but can yield specific products by eliminating from the LOOH the HO^- and the HOO^-, resulting in an internal ketone and an unsaturated compound that has an extra double bond, respectively.

Scission scheme of hydroperoxides (formation of volatile off-flavour compounds)

Hydroperoxides primarily break down through complex scissions and free-radical reactions. Some volatile products, e.g. hexanal, can be formed directly after scissions of hydroperoxides, while some secondary products, such as peroxides, epoxides, hydroxides, dihydroperoxides and ketodienes, can decompose further into various volatile products. Some volatile compounds that have low molecular weights are associated with the typical flavours in foods (Ho and Chen 1994; Paradiso et al. 2009; Schaich et al. 2013).

Scissions of the alkoxyl radicals occur in the termination stage of autoxidation but can also appear in the propagation phase. The amounts and types of the products formed from the scissions depend greatly on the characteristics of the hydroperoxides. A proposed scission scheme for β-scissions of 9-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid (9-HPOD) and 13-hydroperoxyoctadeca-9(*Z*),11(*E*)-dienoic acid (13-HPOD) of linoleic acid to volatile oxidation products was given by Schaich et al. (2013) and Jeleń and Wąsowicz (2012) and is shown in Fig. 2 (Damerau 2015). Briefly, an alkoxyl radical is formed from 9-HPOD by eliminating an •OH group, after which the C-C bond on either side of the carbon atom can be broken (Fig. 2a). In route A, a non-radical product 9-oxo-nonanoic acid is formed, as is a new free radical that can react further with oxygen and result in neutral products and radicals. In this step (route A, Fig. 2a), one product formed is 2-pentylfuran, which commonly occurs in food materials and is considered an important indicator of lipid oxidation. Other products are formed in route B (Fig. 2a), including 8-hydroxyloctanoic acid and octanoic acid. Similarly, an •OH group is removed

from 13-HPOD, leading to an alkoxyl radical (Fig. 2b) whose C-C bond can split on either side of the carbon atom carrying the oxygen atom, resulting in a neutral molecular product, i.e. 13-oxo-9,11-tridecadienoic acid, and an alkyl radical that can further lead to a number of products (route A, Fig. 2b). In route B (Fig. 2b), a typical lipid oxidation product, hexanal, is formed by scission of the C-C bond between the carbon positions C12 and C13. In addition, a radical is formed that reacts further to become neutral products.

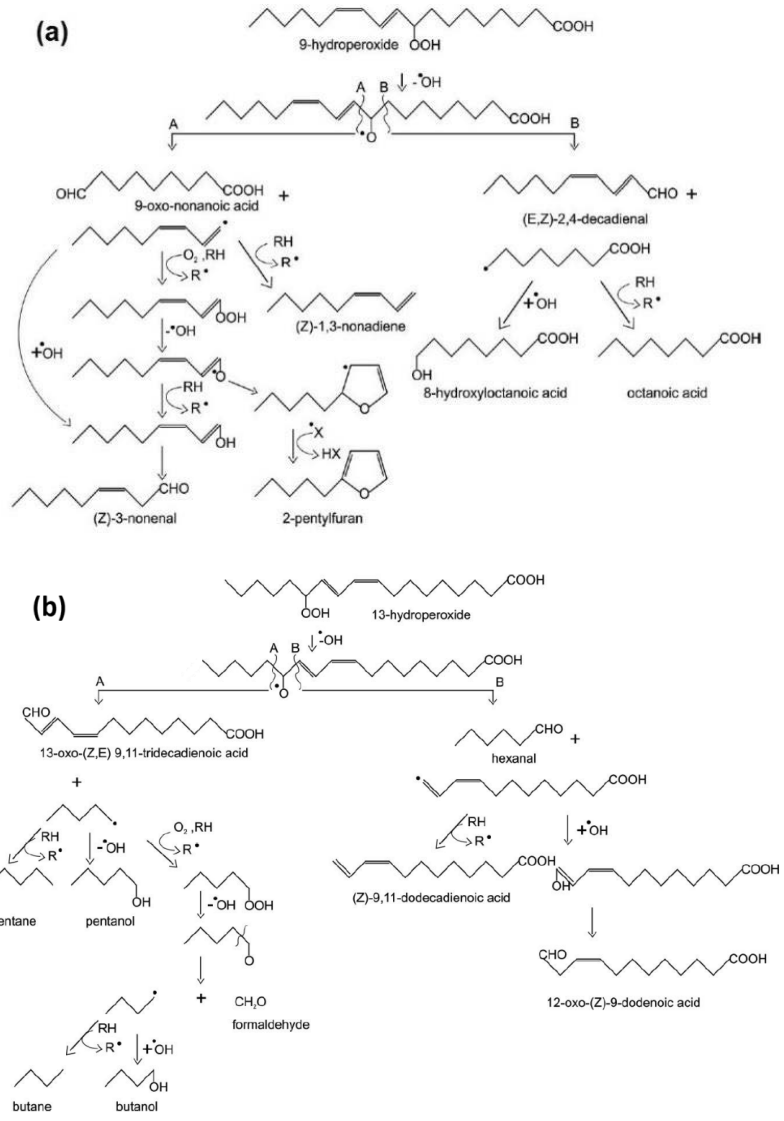


Fig. 2. The β-scissions of (a) 9-HPOD and (b) 13-HPOD from linoleic acid to form lipid oxidation products (Adapted from Damerau (2015), with permission to use).

2.1.1.2 Photo-oxidation

In addition to autoxidation, photosensitised oxidation is another important chemical lipid oxidation pathway that produces hydroperoxides. In this mechanism, lipids are exposed to light in the presence of atmospheric $^3O_2$ and a sensitiser (Frankel 1984). The most important photosensitisers in foods are e.g. chlorophyll, pheophytins, haem pigments and riboflavin. Photosensitised oxidation of UFAs consists of two types. In type I, the reaction occurs between the excited triplet photosensitiser and an LH involving a hydrogen atom or electron transfer, producing a fatty acid radical L•, which reacts with $^3O_2$. Reaction type II mainly includes the formation of reactive singlet oxygen ($^1O_2$) from $^3O_2$. In the presence of an excited photosensitiser, $^3O_2$ is activated by the transfer of energy from the excited photosensitiser, resulting in the formation of $^1O_2$. Unlike in $^3O_2$, with two unpaired electrons in the 2pπ anti-bonding orbitals, in $^1O_2$, one of the 2pπ anti-bonding orbitals has paired electrons, but the other one is totally empty (Choe and Min 2006). The non-radical electrophilic $^1O_2$ reacts easily with compounds that have high electron densities. Therefore, the $^1O_2$ formed reacts directly with the double bonds of UFAs, resulting in unstable peroxide intermediates or an unstable six-membered ring that may decompose quickly and form hydroperoxides, which can be mono-enes or can contain more double bonds that are either conjugated or non-conjugated (Choe and Min 2006). The hydroperoxide isomers thus formed differ somewhat from the hydroperoxides formed in auto-oxidation. For instance, the major products formed from linoleic acid in autoxidation are 9- and 13- hydroperoxides, but two more oxidation products are formed through photosensitised oxidation: 10- and 12- hydroperoxides. The decomposition of hydroperoxides is similar to that in autoxidation, involving complex free-radical chain reactions. Overall, photo-oxidation catalyses a faster lipid oxidation process than autoxidation and thus may lead to a higher potential for lipid degradation in foods (Choe and Min 2006).

2.1.1.3 Control of chemical lipid oxidation in foods

Several methods have been proven to effectively inhibit chemical lipid oxidation, such as decreasing the oxygen content; preventing the exposure of food materials to light; and adding radical scavenging (e.g. tocopherols) or metal chelating antioxidants (e.g. phosphoric acids and citric acid), thereby improving the oxidative stability of foods (Velasco et al. 2010). Many naturally existing compounds have also proven useful in controlling the formation of off-flavour products in foods. A number of naturally occurring antioxidants, such as tocopherols and some other phenolic compounds, may prevent chemical lipid oxidation in foods. Tocopherols reportedly decreased off-flavours during the storage of corn flakes (Paradiso et al.

2008). Also, in corn flakes, the combination of ascorbic acid and tocopherols enhanced the antioxidative stability of lipids more than tocopherols alone or rosemary extracts (Paradiso et al. 2009). Phytochemicals, such as carotenoids and polyphenols, show high antioxidant capacity in plant-derived foods, such as vegetables, fruits and other crops, by scavenging free radicals and $^1O_2$ and chelating metals during the initiation and propagation stages of chemical lipid oxidation (Tsao 2015). In addition, the off-flavours in foods can be masked by some compounds. For instance, a study reported that externally added pea dextrin was suitable for masking the off-flavour resulting from the early stage of lipid oxidation in emulsions (Bottcher et al. 2015).

2.1.2 Enzymatic lipid degradation

Enzymes are crucial substances that act as catalysts in biological systems in all living organisms. Practically, numerous biochemical reactions in living organisms are regulated by enzymes because of their catalytic power and specificity. Most of the known enzymes are proteins, which have a particular reaction region inside them called 'active sites', where the enzymes catalyse the reactions of substrates into various products. Like other catalysts, enzymes accelerate the rates of chemical reactions by lowering the activation energy. Some enzymes catalyse the conversion of substrates millions of times faster than the rates of the corresponding chemical reactions (Berg et al. 2002).

Currently, the use of enzymes in food processing is quite broad and includes germination of cereals to produce wort and beer, bread making, fruit juice production and fruit processing. Enzymes offer many advantages, including regio-, chemo- and stereo-selectivities. In addition, enzymes usually require relatively mild reaction conditions and are quite friendly to environments (Bornscheuer 2018). However, enzymes can also introduce unpleasant problems, including unexpected lipid-derived degradation by endogenous lipid-modifying enzymes in raw food materials. In cereals and legumes, lipid degradation can occur quickly when enzymes come into contact with their substrates during food processing (Lehtinen et al. 2003; Doehlert et al. 2010).

In this thesis, the main focus among lipid-modifying enzymes was on lipase, LOX and POX, which are important for the formation of off-flavours during the processing and storage of plant-derived foods. The special focus was on the properties of these lipid-modifying enzymes in oat and faba bean. In addition, the thesis summarised the knowledge regarding some other lipid-modifying enzymes that occur in cereals and legumes.

2.1.2.1 Lipase

Lipases are ubiquitous enzymes that are widely distributed in nature. Together with esterases, they belong to the carboxyl ester hydrolases, which can catalyse the hydrolysis and/or synthesis of the ester bonds of lipids (Gerits et al. 2014). The 'true' lipases (EC 3.1.1.3), also called triacylglycerol (TAG) hydrolases/lipases, are some of the most important lipid-modifying enzymes that hydrolyse the ester bond(s) of TAGs and thus liberate FFAs from their esters. In this thesis, 'lipase' refers to this type of lipases. In plants, lipases exist mostly in the lipid-reserving tissues of seeds and are activated during germination to hydrolyse the stored TAGs into FFAs, which are further converted into sugars to feed the growth of the seed embryos (Lin et al. 1987). One study that investigated the distribution of lipase in various parts of oat grains (Urquhart et al. 1983) found that 80% of lipase activity was in the bran layers and that activity was reduced by removing the outer bran layer. Similar results were also obtained by Ekstrand et al. (1992), who also reported low lipase activity in the starchy endosperm of oat and that the germination process was capable of enhancing the lipase activity in seed embryos. Although lipase has been isolated from faba bean (Dundas et al. 1978), little is yet known about lipase activity and its effects in faba bean.

Reaction mechanism

Lipases contain a 'lid' that covers the active site, which is called the 'catalytic centre' of the enzyme (Brady et al. 1990). This 'lid' may contain a conformational change at the surface that enables the substrates to enter the 'catalytic centre'. This movement could in turn increase the non-polarity of the surface (Brzozowski et al. 1991). In the presence of non-polar and hydrophobic substances, the lid opens, making the catalytic residues available to substrates and exposing the hydrophobic surface (Jaeger et al. 1999). A three-dimensional structure of a TAG lipase was given by Brady et al. (1990), who reported that the active site of lipase was composed of serine (Ser), aspartate (Asp) and histidine (His). A general reaction mechanism illustrating lipase-catalysed lipid hydrolysis is shown in Fig. 3 (Reis et al. 2009). At the beginning of the reaction, the Ser residue is activated and becomes nucleophilic after deprotonation in the presence of His and Asp (Fig. 3a). Then, the Ser residue attacks the substrate carbonyl group, resulting in an acyl-enzyme intermediate (Fig. 3b). The final step is deacylation, in which a nucleophile attacks the acyl-enzyme intermediate and breaks the ester bond between the acyl component and the Ser residue, resulting in the release of the acyl product and the regeneration of the free lipase enzyme (Fig. 3c).

Lipase can be activated and react quickly in the interface between lipids and aqueous media (Gerits et al. 2014). At the oil-water interface, the hydrophobic head of lipase is bound to the oil by hydrophobic interactions, while the active site of the enzyme is bound to the lipid substrate molecule, for example, TAG or a partially esterified glycerol (Reis et al. 2009). Thereafter, the esterified glycerol molecule is hydrolysed into glycerol and FFAs, and the acyl groups are transformed from TAGs to the water interface. Enzymatic activity, together with the lipids in plants, makes the stability of lipids a major challenge in the food-processing industry. For instance, high lipase activity in oat can greatly promote lipid degradation and cause further problems during processing and storage (Youngs 1978; Matlashewski et al. 1982).

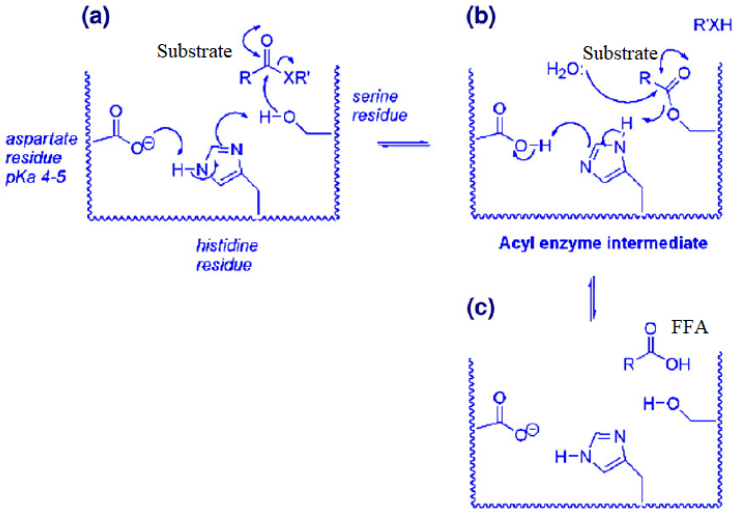


Fig. 3. Reaction mechanism of lipase: a) activation of Ser residue, b) formation of acyl-enzyme intermediate and c) deacylation of the acyl component (Adapted from Reis et al. (2009), with permission to use).

Properties in cereals and legumes

Substrate specificity

Most lipids in plants are present as acylglycerols, which include TAGs, diacylglycerols (DAGs) and monoacylglycerols (MAGs). Lipases may have different lipolysis rates toward different substrates (Jensen et al. 1983). Some lipases show clear specificity in terms of the chain-length of the fatty acids. Unlike esterases, lipases usually prefer long-chain fatty acids (Jensen et al. 1983; Gerits et al. 2014). In addition, lipases can show regio-specificity according to the position of the fatty acid in the glycerol molecule (*sn*-2, *sn*-1 and *sn*-3) (Gerits et al. 2014).

The substrate specificity of oat and wheat seed lipases was studied by Oconnor et al. (1992), who observed no obvious substrate specificity for oat or wheat lipases toward the endogenous TAG substrates, which contained palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid, based on the identical fatty acid composition of the TAGs before and after the reaction. However, in another study, oat grain lipase possessed significant selectivity for TAGs (Piazza et al. 1992). It reacted quickly with TAGs containing oleate, linoleate and linolenate, but slowly with TAGs containing palmitate, stearate and petroselinate. Jung and Moon (2013) investigated the substrate specificity of lipase isolated from oat seedlings using acylglycerol substrates with various numbers of acyl chains. The results showed that the enzyme much preferred monopalmitate to dipalmitate and that only low activity was observed toward tripalmitate. It was suggested that lipase isolated from oat seedlings was more likely to be categorised as esterase or acyltransferase rather than as a TAG lipase. In addition, monoacylglycerols with various acyl chain lengths (butyric acid, lauric acid and palmitatic acid) were compared as substrates (Jung and Moon 2013). Oat seedling lipase showed higher specific activity for short-chain fatty acid substrates than for long-chain ones.

Some studies have investigated the properties of 'true' lipase in faba bean, which has been reported as being single homogeneous esterase that can hydrolyse α-naphthyl acetate, and have reported that faba bean lipase may possess isoelectric and electrophoretic homogeneity similar to some bacteria lipases (Sanclemente and Vadehra 1967; Dundas et al. 1978). Dundas et al. (1978) found that faba bean lipase was more active toward short-chain tributyrin than toward the long-chain TAGs of olive or corn oil. Henderson et al. (1981) demonstrated that the lipase from crude faba bean extract was capable of hydrolysing *p*-nitrophenyl acyl esters of various chain lengths, as well as phosphatidylcholine. However, the substrate specificity of faba bean lipase is still poorly understood.

Optimum reaction pH

Lipases possess activity in a wide range of pH values, from neutral to alkali, as discussed below. The optimum pH for lipase may differ in cereals and legumes, depending on the types and properties of the lipases and the substrates. The knowledge regarding the optimum reaction conditions for faba bean lipase is quite limited, and only a few old studies have investigated its pH requirements (Dundas et al. 1978; Henderson et al. 1981).

One study investigated the activity of oat lipase in a pH range of 5.5–9.0 by measuring the release of FFAs from ^{14}C labelled triolein, finding that lipase from all three oat cultivars studied had an optimum pH of 7.5 (Matlashewski et al. 1982). In addition, one study reported that, when triolein was used as a substrate, the optimum pH for oat lipase reaction was about 7.1–7.3 (Liukkonen et al. 1995). According to a review paper, the optimum reaction pH of oat lipase was 7.4–7.5, while the optimum pH for rice lipase was 7.5–8.0 and for wheat lipase, 7.2–8.2 (Decker et al. 2014). The activity and optimum pH of lipase was measured based on μmol fatty acids released per hour. Unfortunately, this review did not mention the substrate used. Various pH conditions have been also used to measure oat lipase activity. For instance, lipase activity was observed in different parts of oat grains at pH levels 4.6 and 8.0, and the results showed that the enzyme had much higher reaction rates toward 4-methylumbelliferyl heptanoate substrate in alkaline pH than in acid pH (Ekstrand et al. 1992). Other studies have observed lipase activity in oat at pH 7.5 using triolein as a substrate (Qian et al. 2009; Doehlert et al. 2010; Cao et al. 2012). Furthermore, increasing the pH to 8.3 was found to be sufficient to inhibit oat lipase activity, resulting in a low hydrolysis rate when unfractionated oat-oil emulsion was incubated with oat slurries at this high pH (Liukkonen et al. 1995). Dundas et al. (1978) studied faba bean lipase activity at various pH values. Using tributyrin as a substrate, they reported that faba bean lipase possessed activity at relatively the high pH values of up to 8.5. Henderson et al. (1981) also studied the pH requirement of the enzyme, finding that faba bean lipase was active in a pH range of 6.5–9.0, with an optimum pH of 8.5 when measured using *p*-nitrophenyl palmitate (*p*-NPP).

Measuring activity in cereals and legumes

Many analytical methods are available for measuring lipase activity, such as titrimetry, spectroscopy, chromatography, radioactivity and microscopy (Beisson et al. 2000; Gilham and Lehner 2005; Brabcova et al. 2010; Brunschwiler et al. 2013). Like other enzyme activities, lipase activity can be measured using physicochemical methods to monitor the consumption of substrates or the release of products.

The pH-stat is a well-known, continuous-monitoring titrimetric method commonly used to measure lipase activity by adding NaOH to neutralise the FFAs released from the TAG substrates over time in order to maintain a constant pH value (Beisson et al. 2000). Tributyrin is a commonly used and well-accepted substrate for measuring lipase activity, and some long-chain substrates have also been used, e.g. triolein. One study observed faba bean lipase activity

by determining the hydrolysis rate of the TAG emulsion using a pH-stat method. The substrate emulsions contained 5% olive or corn oil or 5–40 mM of tributyrin stabilised with 10% gum Arabic solution (Dundas et al. 1978). The pH-stat method was also used to measure lipase activity from rice brans using 0.2 M of tributyrin substrate emulsion at pH 7.0 (Brunschwiler et al. 2013). The activity (1U) was defined according to the release of titratable butyric acid (μmol/min), and the method was demonstrated as being specific for measuring lipase activity.

Photometric assays are considered time-saving and easy-handling methods compared with the complex and time-consuming traditional titrimetric methods of measuring lipase activity (Vorderwulbecke et al. 1992). Spectrophotometric methods are commonly used to analyse lipase activity. Members of the group of *para*-nitrophenyl esters, which have various carbon-chain lengths, are convenient to use as substrates for lipase assay, which is done by detecting the release of nitrophenol groups based on the absorbance at 410 nm. The *p*-nitrophenyl butyrate (*p*-NPB) is a short-chain substrate that has been used to measure lipase activity in rice bran. A slope increase in absorption at 405 nm during 150 s was acquired, and the activity was given as μmol min^{-1} g^{-1} flour (Brunschwiler et al. 2013). The *p*-NPB substrate was also used to evaluate lipase activity in the extracts from corn seeds (Zhong and Glatz 2006). Furthermore, the long-chain substrates, such as *p*-NPP and *p*-nitrophenyl stearate, were used to measure lipase activity in faba bean (Henderson et al. 1981). That study reported that long-chain *p*-nitrophenyl fatty acyl esters at high concentrations possessed substrate inhibitions toward faba bean lipase, which may be due to the interactions between the long-chain lipid substrates and the enzyme (Galliard 1971). In addition, a spectrophotometric assay using *p*-NPP as a substrate was used to measure the activity of purified lipase from oat seedlings (Jung and Moon 2013). Other photometric methods, such as the dough method, have been used to measure lipase activity in cereals and legumes. The dough method is performed by adding certain amounts of triolein and buffer solution to milled flour samples for incubation and lipid hydrolysis. Then, the released oleic acid is extracted, allowed to react with copper and colour reagents and is converted to copper soaps, which can be detected by spectrophotometry at 715 nm in a process first developed and described by Kwon and Rhee (1986). This method provided an alternative way to measure lipase activity by assaying the FFAs released from their glycerol esters. An older study used the dough method to measure lipase activity from Hinoat grains using radioactive triolein (Matlashewski et al. 1982), and the method was also used to determine the lipase activity during oat malting (Peterson 1999). Similarly, Qian et al. (2009) analysed lipase activity in microwave heat-treated (HT) oat grains using the dough method coupled with the

copper method and triolein as a substrate. In addition, Rose and Pike (2006) used a simple copper-soap method to measure the lipase activity in wheat and wheat bran during storage by assaying the FFAs liberated from olive-oil emulsion.

Chromatography is a technique providing a way to directly analyse the FFAs after the lipolysis of lipid substrates (Gilham and Lehner 2005). The most commonly used methods are gas chromatography (GC) and liquid chromatography (LC). GC is a sensitive method that can quantitatively and simultaneously analyse FFAs after a derivatisation such as methylation. One study used a GC method to study FFAs liberated from triolein and oat oil after incubation with oat slurries: the FFAs were methylated prior to GC analysis, and the lipids were quantified by comparing them with the standards for each lipid class (Liukkonen et al. 1995). Another study observed the activity of purified lipase from oat seedlings using monopalmitate as a substrate; the products were extracted and analysed using a GC method (Jung and Moon 2013). In comparison with GC methods, which usually require a lengthy derivatisation for FFA analysis, LC methods are capable of directly analysing various lipids, such as FFAs, TAGs, DAGs and MAGs. For instance, Lampi et al. (2015) used the normal phase high performance liquid chromatography (NP-HPLC) method to study the release of FFAs in oat flours and extrudates, which in turn could reflect changes in lipase activity during oat processing and storage.

Inactivation in cereals and legumes

Oat grain is unique compared with the grains of some other cereals, partly due to its relatively high content of lipids and soluble dietary fibre (Girardet and Webster 2011). In addition, oat grain has higher lipase activity than some other cereals, such as wheat, rice (Decker et al. 2014) and barley grains (Oconnor et al. 1992). These properties necessitate a milling process for oat that differs from that used for other cereals. In intact oat grains, the enzymes, such as lipase, are physically compartmentalised from the lipid substrates, but when oat grains break down during milling, lipid deterioration begins quickly after the endogenous enzymes come in contact with the substrates (Decker et al. 2014). The most common and efficient approaches to inactivating lipases (or other lipid-modifying enzymes) are heat-denaturation methods, such as kilning (Decker et al. 2014). In oat processing, heat treatment has long been regarded as essential to effectively inactivate endogenous enzymes and obtain oat products that have good lipid stability during storage (Molteberg et al. 1996; Lehtinen et al. 2003). Without this step, oat products may retain a particular raw, bitter taste (Molteberg et al. 1996). In Finland, oat processing usually involves steam treatment at 100 ^{o}C for 2–3 min, during which the moisture of the oat

grains increases from 12–13% to 16–17%, followed by heat treatment at > 95 ºC for > 70 min, after which the moisture content in the oat grains decreases to 13% over 30 min (Salovaara 1993).

As part of studying the inactivation of lipase activity, research has compared several traditional methods. For instance, a comparison of oat grains that had been subjected to dry-kiln treatment before steam treatment and grains that were only steam-treated showed a pronounced difference (Ekstrand et al. 1993). The maximum increase of FFAs in the dry-kiln treated oat samples was about 13–15% after storage for 16 weeks, much lower than in the oat grains not subjected to dry-heat treatment, which had an FFA proportion > 30%. Another study found that oat lipase activity could be controlled using steam preparation, but not the dry-heat treatment (Ekstrand et al. 1992). That study also pointed out that dry-heat treatment itself did not inhibit lipase activity, and thus the subsequent steam treatment was essential and sufficient to fully inactivate the lipase activity in oat grains. One study compared the effects on the storage properties and sensory quality of naked oat using several heat-treatment inactivation methods, including hot-air roasting, normal- or high-pressure steaming, infrared roasting and microwave treatment, and the results showed that all significantly inactivated the lipase activity in oat (Cao et al. 2012). Furthermore, Rose et al. (2008) investigated various methods of inactivating the lipase activity in wheat flour and reported that dry heat for 25 min or microwave heating (1000 W) or steaming for 60 s effectively reduced the lipase activity by 74–96%. The effect of heat treatment on faba bean lipase is reported in only one study, which found that faba bean lipase activity was completely inactivated by exposure to 65 ºC for 2 min (Dundas et al. 1978).

Extrusion treatment has been suggested as an alternative technique for inactivating the enzyme activities in food processing (Singh et al. 2007). It was reported that extrusion of flours from non-heat treated (NHT) oat grains effectively inactivated the native lipase activity and that an extrusion temperature of 70 ºC stabilised the oat lipids (Lampi et al. 2015). Furthermore, microwave heat treatment has been shown to be an effective way of inactivating lipase activity in cereal bran and germ, soybeans (Vetrimani et al. 1992) and naked oat grains (Qian et al. 2009). Microwave heat treatment for 45 s at 850 W was found to inactivate up to 99% of the lipase activity in oat grains (Qian et al. 2009). Likewise, the formation of FFAs in rice bran was reduced by about 90% after microwave heat treatment at 850 W (Ramezanzadeh et al. 1999).

However, heating can also speed the autoxidation of lipids. It was reported that the lower the lipase activity in dried oat fractions, the higher the amounts of lipid volatile-oxidation products detected during prolonged storage (Lehtinen et al. 2003). It was concluded that heat treatment of oat grains primarily accelerated the oxidation of fatty acids in polar lipid fractions, although the formation of FFAs and the lipase activity in these processed oat fractions decreased. Another study reported that increasing the time of microwave heat treatment may increase the amount of hexanal in faba bean (Jiang et al. 2016).

Effects in cereals and legumes in terms of off-flavours

The first reaction in the sequence of lipid deterioration in cereal grains and legumes is usually considered to be lipase-catalysed lipid hydrolysis. Lipid hydrolysis leads to rancidity in foods, which is one of the main negative factors limiting the storage and handling of food products. In addition, the FFAs released can be considered off-flavour compounds (Robards et al. 1988). FFA amounts were shown to be closely related to perceived bitterness in emulsions and to contribute markedly to the generation of off-flavours in soybean lecithins (Stephan and Steinhart 2000). FFA accumulations may increase acidity and reduce pH values in food systems, leading to changes in the foods' functional properties. Furthermore, off-flavour caused by lipase-catalysed lipolysis was found to limit the use of rice bran for human consumption and animal feed (Goffman and Bergman 2003).

2.1.2.2 Lipoxygenase

LOX (EC 1.13.11.12), which belongs to the non-heme iron-containing dioxygenases, is broadly distributed in living organisms. In plants, LOX reactions are important to begin the synthesis of signalling molecules and to induce metabolic or structural changes in cells (Brash 1999). They are responsible for forming hydroperoxides with (*Z*),(*E*)-diene conjugation, preferably by catalysing the oxidation of PUFAs containing (*Z*),(*Z*)-pentadiene moiety in the presence of molecular oxygen (Gardner 1991). In addition, LOX isoenzymes possess various pH optima values, substrates and product specificities. Isoenzymes of LOX have been isolated from the seeds of both cereals and legumes, such as wheat and soybean (Matsui et al. 1992), faba bean (Clemente et al. 2000) and pea (Sanz et al. 1992; Gökmen et al. 2002), while the distribution of LOX in the fractions of cereal and legume seeds remains to be studied.

Reaction mechanism

In plants, LOX has a single polypeptide chain with a molecular mass of *ca.* 94–104 kDa and consist of two domains (Brash 1999): an amino-terminal β-barrel domain and a carboxyl-terminal domain, which consists primarily of α-helices and harbours the iron inside the protein molecule. Usually, LOX enzymes are in a ferrous (inactive) form, but a ferric form is needed for activity and lipid oxidation catalysis (Brash 1999). In plant LOX, the iron at the active site is coordinated with five amino acid residues in the primary crystal structure, including three molecules of His, one carboxyl-terminal isoleucine and His/Asn/Ser (Brash 1999).

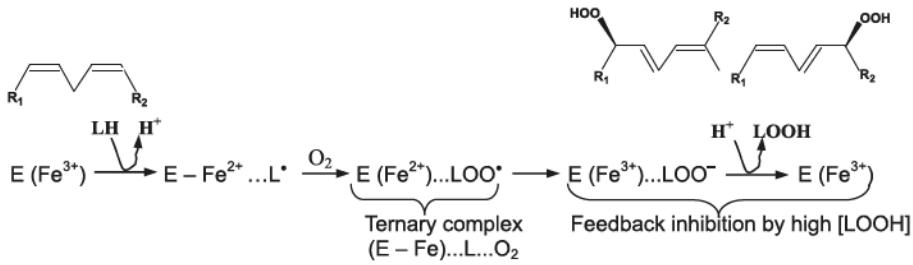


Fig. 4. LOX-catalysed PUFA reaction (Adapted from Schaich et al. (2013), with permission to use).

The most prevalent reaction in plants is dioxygenation, and the substrates are PUFAs with (*Z*),(*Z*)-1,4-pentadiene moiety, such as linoleic and linolenic acids. Fig. 4 shows the LOX-catalysed PUFA oxidation reaction to the formation of conjugated LOOH without generating free lipid radicals (Schaich et al. 2013). The first step in the reaction sequence is the binding of a PUFA to the LOX active site, where the iron is also bound and bisallylic hydrogen removed. Then, the molecular oxygen is bound to the radical, forming a ternary complex. Finally, an H^+ is donated by His residues to complete the formation of an (*E*),(*Z*)-hydroperoxide.

Properties in cereals and legumes

LOX activity among various cereals and legumes varies significantly (Chang and McCurdy 1985; Lehtinen and Kaukovirta-Norja 2011). LOX properties have been studied in some cereals. For instance, wheat LOX was able to produce both 13- and 9-hydroperoxides from linoleic acid substrate, with the latter one accounting for 85% of the total hydroperoxides (Grosch et al. 1976). However, the properties of oat LOX have been poorly studied because LOX activity in oat is quite low compared with that in some other cereals, such as wheat and barley, according to a review paper (Lehtinen and Kaukovirta-Norja 2011). In soybean, the activity and other

properties of LOX have been well studied (Gardner 1989; Schneider et al. 1997; Brash 1999), and soybean has found to usually possess higher levels of LOX activity than some other legumes, such as black bean, chickpea, and faba bean (Chang and McCurdy 1985). Faba bean was found to possess a medium level of LOX activity compared with some other legumes, with a level that lower than that of soybean but higher than that of chickpea or field pea (Chang and McCurdy 1985).

Substrate and product specificities

Both animals and plants contain PUFAs that can be released, especially when the cell structures are destroyed. One study compared the substrate specificity of LOX purified from soybean and wheat seeds using (6*Z*, 9*Z*)-dienoic acids and chain lengths of C_{13}-C_{24} as substrates (Matsui et al. 1992). It found that soybean LOX oxidised a wide range of substrates with chain lengths from C_{15}-C_{24} into hydroperoxides, while the range for wheat LOX was narrower, with only substrates of chain lengths C_{17}-C_{19} being oxidised. However, in addition to PUFA substrates, one type of soybean LOX was found to also oxidise acylglycerols and some phosphoglycerides (Piazza and Nunez 1995). The same study reported that trilinolein was a poor substrate for LOX oxidation, having a reaction rate of only 3% of that of linoleic acid, while diacylglycerols oxidised faster and had a reaction rate 40% that of linoleic acid. It also found that a large portion of the available linoleate of 1,3-dilinolein was converted to hydroperoxide during incubation with soybean LOX (Piazza and Nunez 1995).

The product specificity of LOX in cereals and legumes largely depends on the properties of isoenzymes and reaction conditions. Two isoenzymes of LOX, BBL (broad bean lipoxygenase)-1 and BBL-2, have been purified from faba bean (Clemente et al. 2000). At pH *ca.* 5.6–5.8, BBL-1 produced from linoleic acid both 13-HPOD and 9-HPOD, which had the proportions of 40% and 60%, respectively, as well as 13- and 9-keto-octadecadienoic acids. BBL-2 exclusively produced 13-HPOD and 9-HPOD at the proportions of *ca.* 90% and 10%, respectively. Four types of LOX have been observed in soybean, and they possess different pH optima and product specificities. The 13-HPOD was formed from linoleic acid at pH 9-10 by one isoenzyme of soybean LOX (Shibata et al. 1987), while only 9-HPOD was formed by another LOX isoenzyme whose optimum pH was 6–7 (Gardner 1991). Furthermore, a mixture of 9- and 13-HPOD was detected at pH 6 by two other types of LOX in soybean (Kato et al. 1992). Isoenzymes of LOX have also been studied in some other legumes, such as pea (Yoon and Klein 1979) and chickpea (Sanz et al. 1992). In addition, plant LOX showed pronounced

regio-specificity toward either carbon position C13 or C9 in linoleic and linolenic acids (Gardner 2003). Plant LOX with high pH optima exclusively oxidised the C13 position of linoleic acid at high alkali pHs, resulting in 13-HPOD, while the LOX that had optimum pHs around neutral mainly catalysed oxidation at the C9 position or at both the C9 and C13 positions (Gardner 2003). However, much less is known about the specificity of LOX in oat and faba bean, although a few studies have characterised LOX from faba bean (Alobaidy and Siddiqi 1981; Clemente et al. 2000).

Optimum reaction pH

The pH is one of the most important factors affecting LOX activity. Because various isoenzymes of LOX occur in plants, the optimum pH may vary depending on the source and properties of the LOX. It was reported that faba bean LOX possessed activity in a wide pH range, 4.0–8.0, with the optimum activity occurring at pH 5–6 (Henderson et al. 1981). A later study found that two isoenzymes of faba bean LOX showed activity in a narrow pH range, with their pH optima being 5.6 and 5.8, respectively (Clemente et al. 2000). In green peas, an optimum pH of 6 was determined for LOX activity (Gökmen et al. 2002). In soybean, LOX activity was still present at pHs as low as 4.5, but pHs below 3 could irreversibly inactivate the enzyme (Wang and Hammond 2010). Soybean LOX-1 had optimum activity at pH 9 (Aliasbi et al. 1989), while for two other soybean LOX isoenzymes, the optimum values were both about pH 7 (Chedea et al. 2008). In addition, a study purified LOX from the flours of de-hulled, germinated oat grains and observed that oat LOX possessed activity in a wide range of pH values, *ca.* pH 4–7, with the optimum pH being 4.5 (Yi et al. 2005).

Measuring activity in cereals and legumes

Because hydroperoxides are the main products of LOX-catalysed lipid oxidation, one prevalent method of studying LOX activity is measuring the formation of hydroperoxides from the substrates spectrophotometrically at 234 nm, at which the conjugated dienes of the hydroperoxides have maximum UV absorbance (Gardner 1989; Gökmen et al. 2002; Chedea et al. 2008; Ties and Barringer 2012). Linoleic acid is the substrate most commonly used to determine LOX activity in cereals and legumes. LOX activity and its hydroperoxide products have been well determined using linoleic acid as a substrate in oat (Yi et al. 2005), faba bean (Henderson et al. 1981; Jiang et al. 2016) and some other legumes, such as soybean, pea and black bean (Chang and McCurdy 1985; Gökmen et al. 2005). This method was also used to measure LOX activity in 14 legumes (Chang and McCurdy 1985). In addition, Hamberg and

Hamberg (1996) reported that the LOX activity in cereal seeds, such as oat, rye and barley, could be measured using a radio thin-layer chromatography (radio-TLC) method and ^{14}C labelled linoleic acid and the LOX activity expressed as nmol oxygenated derivatives (30 min)$^{-1}$ mg^{-1} protein. Another reliable, sensitive method of measuring soybean LOX activity was based on oxygen consumption during the formation of hydroperoxides (Gardner 1989). However, these methods can provide only an estimate of total hydroperoxide formation, because they are unable to determine the identity and quantity of individual hydroperoxides. Nevertheless, this knowledge is important, because different hydroperoxide isomers produce different degradation products. Thus, it is crucial to learn more about the individual hydroperoxides formed from LOX-catalysed lipid oxidation by e.g. HPLC, and to further investigate the properties of LOX in cereals and legumes.

Inactivation in cereals and legumes

LOX activity can be inhibited by chemical inhibitors or through food processing methods. In nature, several types of enzyme inhibitors can inhibit LOX activity, mainly including substrate 'suicide' inhibitors, such as colneleic acid; chain-breaking antioxidants and iron chelators; and active-site or active-site iron inhibitors (Nieuwenhuizen et al. 1997; Pham et al. 1998; Wang and Hammond 2010). In food processing, the most commonly used method of inactivating LOX in cereals and legumes is heat treatment.

Heat treatment is a traditional, simple, effective way to inactivate LOX activity and prevent undesirable reactions in cereals and legumes, such as rice and soybean (Wang and Hammond 2010). In addition, microwave treatment was used to inactivate LOX activity in cereal bran and germ and soybean (Vetrimani et al. 1992). A pre-treatment of microwave heat was used to inactivate LOX activity in faba bean (Jiang et al. 2016). It was reported that microwave heating of faba beans at 950 W for 1.5 min was an optimum pre-treatment that effectively inactivated the endogenous LOX activity. In addition, steam treatment for 2 min was found to inactivate LOX activity in soybean (Wang and Hammond 2010). An ultrasonic cavitation method was used to inactivate LOX activity in whole soybean-flour suspensions (Thakur and Nelson 1997), and an ultrasonic treatment of 20 kHz at pHs 5.0 and 4.0 decreased the activity by 70–85%. In addition, some other methods, such as ionising irradiation, adding alcohol, ultrasonic and pressure treatments, were used in combination with heat treatment to inactivate soybean LOX (Lopez et al. 1994; Lopez and Burgos 1995).

Effects in cereals and legumes in terms of off-flavours

LOX catalysed lipid oxidation is one of the most important factors affecting the shelf-life of food products, especially if the enzymes had not been properly inactivated during processing. It gives rise to a series of unhealthy compounds in foods, such as active aldehydes and free radicals. The hydroperoxides formed are unstable and may decompose into various volatile off-flavour compounds, such as hexanal, as well as non-volatile products. In addition, the hydroperoxides formed can be used by POX to form non-volatile products (*Section 2.1.2.3*).

Cereals, such as wheat, rice (Orsavova et al. 2015) and oat (Leonova et al. 2008), usually contain PUFAs, which enable lipid oxidation by the LOX and further formation of off-flavour products. Molteberg et al. (1996) found a significant difference in the levels of volatile compounds in oat after 5 and 42 weeks of storage, and suggested that the term 'rancid' was related to high levels of 2-pentylfuran, 1-hexanol and 2-heptanone and to low levels of octanal and 1-octen-3-ol. Galliard and Gallagher (1988) found that off-flavour in wheat was strongly associated with LOX-catalysed fatty acid peroxidation. In legumes, LOX-catalysed lipid oxidation is believed to be associated with undesirable off-flavours, such as the so called 'beany flavour' in faba bean (Roland et al. 2017). A large number of volatile compounds belonging to various categories, such as aldehydes, alcohols, alkanes, ketones and aromatic hydrocarbons, were identified and suggested as being responsible for the undesirable flavours in faba bean (Oomah et al. 2014). One study indicated a correlation between LOX activity and the occurrence of a bitter taste in soybean during maturation (Rackis et al. 1972). Therefore, LOX can greatly influence the formation of off-flavour compounds in both cereals and legumes.

2.1.2.3 Peroxygenase

POX (EC 1.11.2.1), which catalyses the hydroperoxide-dependent conversion of UFAs into non-volatile flavour compounds, such as epoxy and hydroxy fatty acids, has been found in higher plants (Hamberg and Hamberg 1996). The importance of epoxy fatty acids is pronounced because they are crucial intermediates for the biosynthesis of defence compounds and cutin monomers in plants (Blée and Schuber 1990). Unlike cytochrome P-450-dependent enzymes and peroxidases, POX is a unique heme-containing mono-oxygenase enzyme that does not require molecular oxygen (Hanano et al. 2006) and accepts only hydroperoxides as oxygen donors (Blée et al. 1993).

POX activity has been detected broadly in cereals and legumes, such as oat (Hamberg and Hamberg 1996; Piazza et al. 1999; 2001), wheat, rye, barley (Hamberg and Hamberg 1996), pea (Ishimaru and Yamazaki 1977) and soybean (Blée et al. 1993). Three types of POX (*AsPXG 1, AsPXG2* and *AsPXG3*) were identified in oat using a gene-encoding method, and *AsPXG 1* and *AsPXG 3* in particular were shown to be typical of POX because they efficiently catalysed the epoxidation of UFAs in the presence of cumene hydroperoxide as an oxygen donor (Meesapyodsuk and Qiu 2011; Benaragama et al. 2017). However, the functionality and properties of POX, as well as its role in forming off-flavours from lipid oxidation in oat and faba bean, are poorly understood. The knowledge regarding POX in faba bean is especially limited.

Reaction mechanism

POX has been suggested as having two reaction mechanisms (Fig. 5). One, Reaction A (Fig. 5), is suggested as explaining the intermolecular oxygen-transfer reactions by plant POX that mediates co-oxidative reactions, including epoxidation, sulfoxidation and hydroxylation (Hanano et al. 2006). POX mediates the cleavage of the hydroperoxide and then yields an alcohol and ferryl-oxo complex. Thereafter, an oxygen atom from the intermediate is transferred to an acceptor molecule bound to the active site of the POX. Meanwhile, POX catalyses co-oxidations, e.g. the epoxidation of UFAs (Hanano et al. 2006). A reaction mechanism for soybean POX was proposed using 13-hydroperoxyoctadeca-9(*Z*),11(*E*),15(*Z*)-trienoic acid (13-HPOT) (Blée et al. 1993). This mechanism supports that the ferryl-oxo intermediary complex, created by the heterolytic O-O bond cleavage of 13-HPOT, can act as an oxygen donor for substrates.

The other POX-catalysed lipid oxidation reaction mechanism, Reaction B (Fig. 5), was called an intramolecular oxygen transfer involving heterolytic cleavage of the hydroperoxide (Blée et al. 1993; Hanano et al. 2006). When the reaction begins, an oxygen molecule is cleaved from the hydroperoxide to the enzyme, yielding a hydroxy fatty acid and a ferryl-oxo intermediate. The ferryl-oxo intermediate formed prefers to epoxidise the *cis*-double bond and yields an epoxy-hydroxy fatty acid (Reaction B, Fig. 5). Blée et al. (1993) illustrated the epoxidation of 13-hydroxyoctadeca-9(*Z*),11(*E*),15(*Z*)-trienoic acid (13-HOT) from 13-HPOT by intramolecular oxygen transfer, resulting in the formation of a ferryl-oxo complex that then further epoxidised the 15,16(*Z*)-double bond of the 13-HOT, forming 15,16-(*Z*)-epoxy-13-

hydroxyoctadeca-9(*Z*),11(*E*)-dienoic acid. Furthermore, oat POX further converted the epoxy and epoxy-hydroxy fatty acids to di- and tri- hydroxy fatty acids (Hamberg and Hamberg 1996).

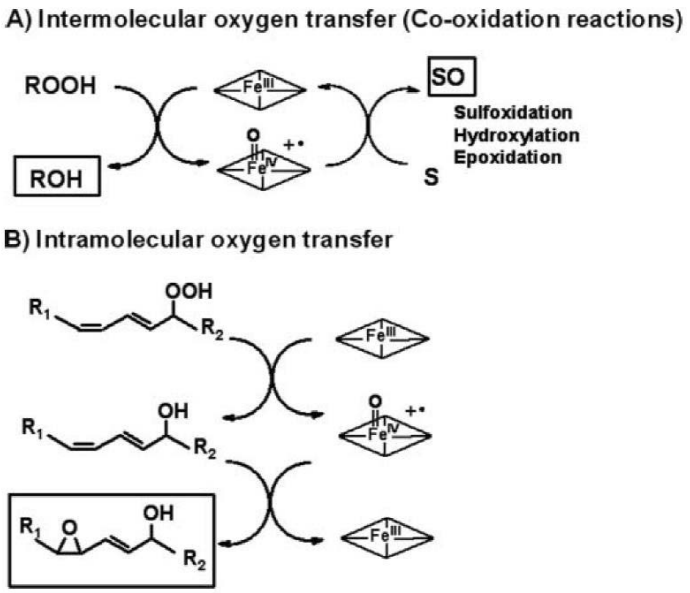


Fig. 5. Mechanism for the formation of (epoxy-)hydroxy fatty acids catalysed by plant POX from hydroperoxides (Adapted from Hanano et al. (2006), with permission to use).

Properties in cereals and legumes

Substrate and product specificities

The activity of POX toward different substrates varies markedly. When TAG and DAG substrates were incubated with ground oat grains in a buffer mixture, no epoxy products were formed, which indicated that TAG and DAG were not substrates for oat POX (Piazza and Foglia 2006). POX prefers to catalyse the reactions of UFAs. One study used a variety of monounsaturated FFAs with various carbon chain lengths (14–24 carbon chains) and PUFAs (linoleic and linolenic acids) to determine the substrate specificity of the oat POX *AsPXG3* (Benaragama et al. 2017). Oat POX *AsPXG3*, obtained using a cloning method from oat grain genes, catalysed the reactions of both monounsaturated fatty acid and PUFA substrates, using linoleic acid as the most preferred substrate. When oleic and elaidic acids were used as substrates to study the selectivity of oat POX, oleic acid was found to be preferred as a substrate to its *trans* form, elaidic acid (Piazza et al. 2000). However, another study found that oat POX (*AsPXG1*) catalysed only the epoxidation of UFAs that had the *cis* configuration, while the

trans configuration of the UFAs and the saturated fatty acids were not accepted as substrates by this enzyme (Meesapyodsuk and Qiu 2011).

Oat grain POX incubated with ^{14}C-labeled linoleic acid produced several different products (Hamberg and Hamberg 1996), mainly epoxy and hydroxy fatty acids, including 9-hydroxy-10,12-octadecadienoic acid, 9,10-epoxy-12-octadecenoic acid, 12,13-epoxy-9-octadecenoic acid, 12,13-dihydroxy-9-octadecenoic acid and 9,12,13-trihydroxy-10-octadecenoic acid. Furthermore, the epoxy and hydroxy fatty acid products have also been produced by POX in legumes. A study reported that only the *cis*-double bonds in UFAs yielded *cis*-epoxy fatty acids and that POX epoxidised only the 15,16-(*Z*) double bond but not the conjugated diene of 13(*S*)-hydroperoxyoctadeca-9(*Z*),11(*E*),15(*Z*)-trienoic acid, which indicated the high regio-selectivity of soybean POX (Blée et al. 1993). According to this study, 13-hydroxyoctadeca-9,11,15-trienoic acid and 15,16-epoxy-13-hydroxyoctadeca-9,11-dienoic acid were formed from the reaction of 13-hydroperoxyoctadeca-9,11,15-trienoic acid with solubilised, partially purified soybean POX.

Optimum reaction pH

An optimum pH for POX activity from oat grains was identified by reacting sodium oleate as a substrate with various oxidants. The optimum pH was determined to be 7.5 when *t*-butyl hydroperoxide was used as the oxidant and 5.5 when hydrogen peroxide was used (Piazza et al. 2001). A range of pH conditions was used to evaluate the synthesis of epoxy fatty acids by oat POX, and large amounts of FFAs were converted into epoxides at pH 6–7, with the optimum value being pH 7 (Piazza and Foglia 2006). To date, few studies are available on the optimum pH reaction conditions for POX activity in plants, but most studies of fatty acid epoxidation using oat POX were conducted around neutral pH, 6–7 (Hamberg and Hamberg 1996; Piazza et al. 1999; Meesapyodsuk and Qiu 2011; Benaragama et al. 2017).

Measuring activity in cereals and legumes

Many studies involving POX activity measurement have observed the reactions of UFAs to oxidants or fatty acid hydroperoxides based on the formation of products (Piazza et al. 1999; 2003a; Fuchs and Schwab 2013). Choosing a proper oxidant and reaction pH is important to determine POX-catalysed reactions and activities. Several oxidants, such as *t*-butyl hydroperoxide, cumene hydroperoxide and hydrogen peroxide, have been used to measure POX activity and to produce epoxy fatty acids by POX (Piazza et al. 2000; 2001). POX activity has

been measured in a narrow pH range around neutral and using a buffer-emulsion reaction system (Piazza et al. 2000; Piazza et al. 2002; Huang and Schwab 2012; Fuchs and Schwab 2013; Benaragama et al. 2017). One old study used a hydroxylation method to measure pea POX activity in a phosphate buffer phase at pH 7.2 by converting indole into indoxyl, which yielded maximum absorption at the UV wavelength 380 nm (Ishimaru and Yamazaki 1977). Another substrate, aniline, was used to measure POX activity during maize germination (Lequeu et al. 2003). Hamberg and Hamberg (1996) assayed POX activity by measuring the formation of 9,10-epoxystearate with a radio-TLC method and expressed the POX activity as nmol 9,10-epoxystearate $(10\ min)^{-1}\ mg^{-1}$ protein formed in the reaction. In addition, an HPLC method coupled with an evaporative light scattering detector (HPLC-ELSD) was used to study the enzyme activity of a microsomal fraction containing oat POX by measuring the proportions of the products from the epoxidation reactions of oleic acid and elaidic acid (Piazza et al. 2000).

The source and type of oxidants can markedly affect POX activity and the production of epoxides. One study investigated the influence of oxidants and solvent media on the epoxidation of oleic acid using membrane supported POX from oat grains (Piazza et al. 2000). It found that *t*-butyl hydroperoxide provided the highest epoxide yield (79%), followed by cumene hydroperoxide (56%). In addition, a higher epoxide yield was obtained in an aqueous medium than in a heptane medium. The yield was clearly lower with urea-hydrogen peroxide (20%) and hydrogen peroxide (17%). Another study compared the oxygen donors *t*-butyl hydroperoxide and hydrogen peroxide in the epoxidation of oleic acid (Piazza et al. 2001). About 80% of oleic acid was converted in the presence of *t*-butyl hydroperoxide incubated for 24 h with oat seed POX, while the value was only 33% when hydrogen peroxide was used, and it was suggested that hydrogen peroxide might deactivate POX activity. In addition, the 13-hydroperoxyoctadeca-9,11,15-trienoic acid was found to act as an oxygen donor, which can oxidise oleic acid into epoxide, but the reaction rate was less efficient than for cumene hydroperoxide (Blée et al. 1993).

Since POX activity is associated with the formation of non-volatile lipid oxidation products in cereals and legumes, to better understand the activity and properties of POX, it is important to develop a method to reliably analyse the products of POX-catalysed lipid oxidation, such as epoxy and hydroxy fatty acids.

Inactivation in cereals and legumes

The activity of plant POX can be inhibited by chemical inhibitors, but in food production, the processing methods should be able to inactivate POX. Plant POX is very sensitive to some inhibitors, such as β-mercaptoethanol and the organophosphorus terbufos (Hanano et al. 2006). β-mercaptoethanol was found to act as a competitive inhibitor at 1 mM, and terbufos was called a 'suicide substrate' that effectively inactivated POX activity even at the low concentration of 3 mM. Other inhibitors studied include tetcyclacis, which strongly inhibited the POX activity in tomatoes (Aghofack-Nguemezi et al. 2011), and organophosphorothioate, which dramatically decreased the epoxide content and inhibited the activity of maize POX (Lequeu et al. 2003).

As is the case with lipase and LOX, POX activity can be inactivated using heat treatment during food processing. However, only one study has investigated the influence of heat treatment on inactivating POX activity by measuring the formation of products. Doehlert et al. (2010) stored both oat grains and flour at 37 ^{o}C for up to 22 weeks to compare the effect of steaming and roasting treatments on the formation of epoxy and hydroxy fatty acids. The steaming was conducted in a vegetable steamer for 20 min, and the roasting was carried out at 106 ^{o}C for 120 min. The relative humidity at which the oat grains and flour samples were stored was 65%. The results showed that heat treatment greatly reduced the formation of epoxy and hydroxy fatty acids, indicating that oat POX activity was successfully inhibited by such treatments.

Effects in cereals and legumes in terms of off-flavours

Formation of lipid-derived, non-volatile compounds should be prevented, because they can lead to undesirable off-flavours in plant-derived foods, thereby decreasing customer acceptance of those foods (Baur et al. 1977; Biermann et al. 1980; Doehlert et al. 2010; Simsek and Doehlert 2014). Epoxy and hydroxy fatty acids have been suggested as significantly affecting the sensory properties of food products. The occurrence of bitterness in soybean has been reported as closely related to the main compounds 9,10,13-trihydroxyoctadec-11-enoic and 9,12,13-trihydroxyoctadec-10-enoic, which have taste thresholds in the range from 0.6–0.9 μmol/ml (Baur et al. 1977). Sensory analysis of hydroxy fatty acids, such as 9-hydroxy-10,12-octadecadienoic acid and 13-hydroxy-9,11-octadecadienoic acid, have suggested that they are associated with strong bitterness in oat (Biermann et al. 1980), but further studies are needed to investigate the role of epoxy and hydroxy fatty acids in bitterness in foods.

2.1.2.4 Other lipid-degrading enzymes in plants

In addition to the enzymes studied in this thesis, some other lipid-modifying enzymes also play important roles in the degradation of lipids and the formation of off-flavour compounds. Hydroperoxide-degrading enzymes are responsible for the scission of hydroperoxides and their further reactions to produce various volatile products responsible for off-flavours in plants (Ho and Chen 1994). These enzymes include hydroperoxide lyase (HPL), hydroperoxide dehydrase, alcohol dehydrogenase (ADH), isomerase, hydroperoxide isomerase and divinyl ether synthase (Fauconnier and Marlier 1997; Grechkin 2002). Furthermore, this thesis summarises some lipid-hydroxylation enzymes because hydroxy fatty acids are non-volatile off-flavour compounds that can cause a bitter taste in foods, such as in oat and wheat (Biermann et al. 1980), and have also been used as new products for industrial applications (Hou 2000). These lipid-hydroxylation enzymes for generating hydroxy fatty acids in plants include P450 mono-oxygenases, hydroxylase and hydratase (Bornscheuer 2018; Demming et al. 2018).

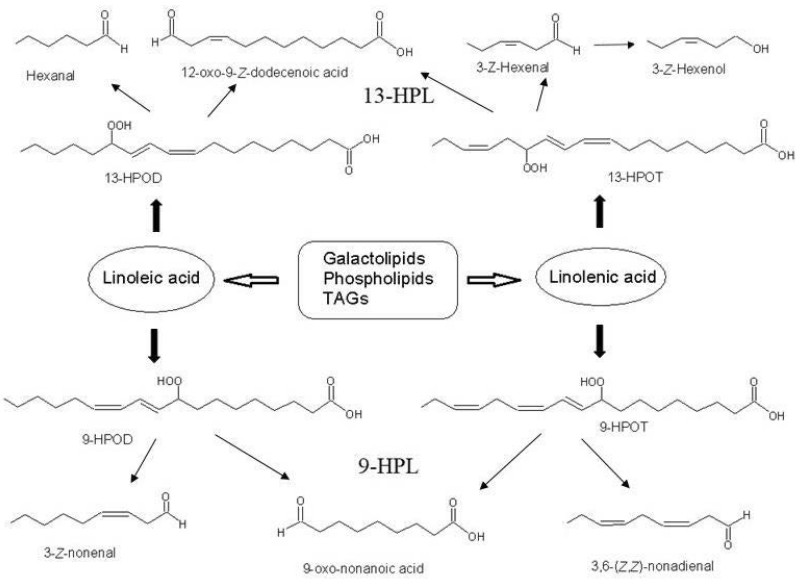


Fig. 6. HPL-catalysed hydroperoxide degradation (Figure combined from Fauconnier and Marlier (1997) and Gigot et al. (2010)).

HPLs (EC 4.1.2.-) are hydroperoxide-dependent enzymes that catalyse scission of hydroperoxides into ω-oxo-acids and aldehydes (Fig. 6). HPL has been found to be broadly distributed in plants, such as cucumber, tomato and soybean, and in plant leaves (Fauconnier and Marlier 1997; Matsui et al. 2000; Froehlich et al. 2001). The presence of HPL has not yet

been confirmed in cereals and faba bean, but it has been assumed to be abundant based on an observation of typical products (Lehtinen and Laakso 2004). Plant HPLs possess activity in a wide range of pH conditions. For instance, the HPL in soybean cotyledons and tea leaves was found to have activity at neutral pH and that of cucumber cotyledons at pH 8 (Fauconnier and Marlier 1997). In addition, plant HPLs have different specificities toward substrates, according to which the enzyme was classified into two groups (Noordermeer et al. 2001). The 9-HPL exclusively cleaves 9-isomers of hydroperoxides, leading to formation of 9-oxononanoic acid from both 9-HPOD and 9-hydroperoxilinolenic acid (9-HPOT) and other compounds, such as (3*Z*)-nonenal from 9-HPOD and (3*Z*,6*Z*)-nonadienal from 9-HPOT (Fig. 6). The 13-HPL specifically cleaves hydroperoxide 13-isomers, resulting in formation of 12-oxo-(9Z)-dodecenoic acid from 13-HPOD and 13-HPOT. Hexanal is typically produced from 13-HPOD, but for 13-HPOT, the special product is (3*Z*)-hexenal (Fig. 6). HPL activity is usually associated with isomerase activity (Gigot et al. 2010), which transforms the structure of (*Z*)-isomer into (*E*)-isomer. For instance, (*Z*)-3-hexenal could be reversible transferred into (*E*)-2-hexenal in the presence of isomerase.

ADH (E.C. 1.1.1.1), which belongs to the multigene family, plays an important role in plants, including in growth and development, aroma formation and fruit ripening (Jin et al. 2016). Generally, ADH comprises a group of isoenzymes that oxidises the primary and secondary alcohols into aldehydes and ketones, respectively. In plants, these reactions are reversible, and the aldehyde products degraded from hydroperoxides are converted by ADH into corresponding alcohol compounds (Hatanaka 1993). For instance, hexanal, (*Z*)-hex-3-enal and (*E*)-hex-2-enal formed by HPL are further converted by ADH into hexan-1-ol, (*Z*)-hex-3-en-1-ol and (*E*)-hex-2-en-1-ol. ADH has also been detected in legumes, such as faba bean (Leblova 1974) and soybean (Leblova and Perglerova 1976), but generally its activity in plants is not high (Gigot et al. 2010). ADH activity is usually determined in a reverse reaction by converting ethanol substrate into acetaldehyde while the concomitant reduction of NAD^+ to NADH occurs (Fibla and Gonzalezduarte 1993). ADHs may have different optimum pH values depending on their types and properties. The optimum pH for the ADH isolated from faba beans was found to be 8.7 (Leblova 1974), soybean ADH catalysed alcohol oxidation at an optimum pH of 8.7, and the optimum pH for reducing acetaldehyde was found to be 7.1 (Leblova and Perglerova 1976).

Hydroperoxide dehydrase (E.C. 4.2.1.92) is another enzyme that exists in plants and includes hydroperoxide isomerase and hydroperoxide cyclase (Zimmerman 1966; Fauconnier and

Marlier 1997). Hydroperoxide isomerase was found in both cereals and legumes, such as wheat germ, barley and soybean seeds, and it catalysed the transformation of conjugated diene hydroperoxides formed from linoleic and linolenic acids into monoenoic α- and γ- ketols (Zimmerman and Vick 1970), and hydroperoxide cyclase transformed 13-HPOT into 12-oxo-phytodienoic acid (Fauconnier and Marlier 1997). Hydroperoxide dehydrase used both the 9- and 13-hydroperoxides from linoleic and linolenic acids as substrates, and the optimum pH for its reaction is 6–7 according to a review paper (Fauconnier and Marlier 1997).

P450 mono-oxygenases are enzymes that catalyse the transfer of oxygen atoms from molecular oxygen into fatty acids in the presence of NADPH and thereafter form hydroxy fatty acids. P450 has been classified into two types: α-hydroxylases (EC 1.11.2.4) and ω-hydroxylases (EC 1.14.15.3) (Van Bogaert et al. 2011). The α-hydroxylases are carboxyl-terminal hydroxylases, and ω-hydroxylases are terminal/subterminal hydroxylases that are present in plants (Kim and Oh 2013). Both α- and ω- hydroxylase have specificities regarding substrates and regions. Palmitic acid was ω-hydroxylated by incubating it with faba bean microsomes (Soliday and Kolattukudy 1977). A mid-chain hydroxylase was determined to exist in germinated faba bean seeds because 16-hydroxypalmitic acid was hydroxylated on its carbon positions C9 and C10 and further formed dihydroxypalmitic acid (Soliday and Kolattukudy 1978). Furthermore, hydroxylases have been observed in some other cereals and legumes, such as wheat (Zimmerlin and Durst 1992) and *Vicia sativa* (Pinot et al. 1992).

2.2 Analysis of lipid degradation products related to off-flavours in cereals and legumes

2.2.1 Analysis of volatile lipid oxidation products using the HS-(SPME)-GC-MS method

Measuring volatile off-flavour compounds and aromas usually requires sensitive techniques, because their quantities are generally small. Recently, headspace solid-phase micro-extraction coupled with GC and mass spectrometry (HS-SPME-GC-MS) has been popularly used to study the lipid oxidation products in various types of foods. It is considered to be a fast, sensitive, efficient method of monitoring lipid oxidation to analyse volatile flavour compounds. The HS-SPME technique is based on the absorption and adsorption of volatile compounds from samples into a coated silica fibre, followed by desorption of analytes in a hot-injection port and GC analysis (Jelen et al. 2012). However, the effectiveness of SPME is influenced by several parameters, such as the fibre type, the volume of the sample, temperature, extraction time, modes of extraction and analyte desorption (Wardencki et al. 2004). In addition, the competition

for space of the analytes on the fibre is also one important factor that affects the effectiveness of SPME (Coleman 1996; Roberts et al. 2000; Thomsen et al. 2016).

Wardencki et al. (2004) reviewed many types of SPME fibres, including polyacrylate, divinylbenzene-carboxen-polydimethylsiloxane (DVB/CAR/PDMS), PDMS-DVB and CAR-PDMS, used for volatile analysis from food matrices. A PDMS fibre with a thickness of 100-μm is quite often used to analyse fragrances and contaminants in food products. For instance, when various types of SPME fibres were compared to study the changes of volatile profiles in processed oat, a PDMS fibre with a thickness of 100-μm was found to be the most suitable, based on the compounds collected and the response of the peak area (Sides et al. 2001). Volatile compounds from lipid oxidation were analysed using HS-SPME with DVB/CAR/PDMS fibre and GC coupled with flame ionisation detector (GC-FID) to study the effects of polar compounds, mainly including phospholipids, FFAs, sterols and hydrophilic phenols, on the formation of volatiles in virgin olive oils (Koprivnjak et al. 2009). A similar HS-SPME-GC/MS method using the same extraction fibre was used to investigate the volatile compounds from lipid oxidation in oat samples after extrusion treatment (Lampi et al. 2015). An SPME method together with GC-olfactometry was used to investigate the flavour of oat flakes after heat treatment. Methyl-3-furanthiol, 1-octen-3-ol, methional, 2-methyl-3,5-diethylpyrazine and dimethyl trisulfide were identified as the major compounds (Klensporf and Jeleń 2008). Cognat et al. (2012) used SPME-GC-MS to identify in oatcakes the chemical classes and origins of the compounds related to rancidity or oat flavour, including furans, alkanes, ketones, aldehydes and alcohols, and a wide range of compounds was detected with fairly good reproducibility. In addition, the relationship between volatile profiles and flavour development in an oat-based biscuit was investigated using a static headspace method and a GC-MS method (Cognat et al. 2014). Furthermore, the identity and quantity of volatile oxidation products were studied from spray-dried emulsions using different HS-SPME conditions (Damerau et al. 2014). The results demonstrated that the method was able to examine the matrix-related changes in foods in terms of the release of volatile compounds. The HS-SPME-GC/MS method was also used to identify 45 volatile compounds in Canadian-grown faba bean cultivars, mainly including alcohols, ketones and aldehydes (Oomah et al. 2014). Finally, a recent study used this method to evaluate volatile flavour profiles in native faba bean that had low/high tannin contents (Akkad et al. 2019).

The HS-SPME-GC/MS method has been shown to be an effective approach to analysing the volatile lipid oxidation compounds in food materials to identify lipid oxidation. However, it is important to optimise the analytical conditions, because many factors can affect the results.

2.2.2 Analysis of non-volatile oxidised fatty acids (NVOFAs) by chromatographic methods

2.2.2.1 Extracting NVOFAs from foods

Various methods have been used to extract lipids and lipid-degradation components from food matrices, such as soxhlet extraction, sonication extraction, microwave dissolution and supercritical fluid extraction. Traditionally, solvent extraction of the lipids and lipid oxidation products is often used in lipid analysis. A chloroform-methanol-water solvent-extraction method was used to extract the lipids and lipid oxidation products from oat (Doehlert et al. 2010). A similar method having a chloroform-methanol solvent was used to extract lipids and oxidised fatty acids, including hydroxy and epoxy fatty acids, from oat (Leonova et al. 2008). However, it is difficult to extract lipids, particularly from some extruded food products, because lipids can be entrapped and bounded in complex matrices with e.g. starch and proteins (Moisio et al. 2015). Because most lipid oxidation products are unstable and do not tolerate high temperatures, extraction methods should be able to efficiently extract lipids from foods as gently as possible without degrading the lipid oxidation products.

Accelerated solvent extraction (ASE) is a method that uses high temperature and pressure to accelerate penetration of solvents into food samples. ASE has been described as providing a platform that enabled better recovery of the analytes (Richter et al. 1996). A study was conducted to compare the lipid yield from *Amaranthus* spp. seeds using an ASE method and the standard soxhlet extraction (Kraujalis et al. 2013). The results showed that the two extraction methods provided similar yields of lipids but that the ASE extraction was much faster than the soxhlet one. Many studies have used an ASE method to extract lipids from various sample materials (Richter et al. 2004; Quénéa et al. 2012; Tang et al. 2016). However, extraction of lipid-derived oxidation products, such as hydroperoxides, has been reported in only one study. A gentle ASE method for extracting the oxygenated fatty acids from dry extruded pet food was developed (Yao and Schaich 2014). The results of the extraction showed that the hydroperoxide decomposition and oxidation of lipids were minimal at 40 ^{o}C but that higher temperatures decomposed the oxidation products, thereby reducing their yield. Thus, it was suggested that the extraction temperature be limited to 40 ^{o}C and that the lipids be extracted

using combinations of polar solvents: a mixture of chloroform-methanol 2:1 (v/v) and one of hexane-methanol 2:1 (v/v).

2.2.2.2 Analysing non-volatile oxidised fatty acids

GC-FID has frequently been used to analyse lipid-derived off-flavour compounds. The methods used for hydroxy and epoxy fatty acids have mainly involved applying GC-FID or GC-MS (Jenske and Vetter 2007; Doehlert et al. 2010; Meesapyodsuk and Qiu 2011; Simsek and Doehlert 2014; Benaragama et al. 2017). The derivatisation of FFAs usually requires a high temperature, which should be avoided, because the oxidised fatty acids decompose easily during heat treatment. Leonova et al. (2008) introduced a GC-MS method of analysing epoxy and hydroxy fatty acids from oat-lipid extract after methylation by diazomethane using methyl 17-hydroxystearate as an internal standard. The study reported that GC-MS performed better after the hydroxylated fatty acid methyl esters were converted into trimethylsilyl derivatives. Another study introduced an acid-catalysed esterification of total fatty acids by reaction with sulfuric acid in methanol at 50 ^{o}C (Doehlert et al. 2010), after which all the free-hydroxyl groups of methylated lipids were silylated using a mixture of pyridine, hexamethyldisilazane and trimethylchlorosilane and then subjected to GC-FID/MS determination.

However, to analyse non-volatile lipid oxidation products from FFAs, GC methods usually require a lengthy time to prepare samples and extensive processing for sample derivatisation. Thus, due to their high accuracy and efficiency and less complex sample preparation, LC methods are being used more frequently to analyse non-volatile lipid oxidation products, such as epoxy and hydroxy fatty acids (Santiago-Vazquez et al. 2004; Orellana-Coca et al. 2005; Levandi et al. 2009; Yang et al. 2013; Yuan et al. 2018). A descriptive, rapid method was developed for analysing epoxides, including regio-isomers, using HPLC-ELSD (Orellana-Coca et al. 2005). Using this method, unsaturated fatty acids and their epoxidation products were separated using a reversed phase C-18 column with a mobile phase of methanol-water and 0.05% acetic acid. In addition, lipid oxidation compounds produced by enzymes were extracted and analysed using an LC method equipped with a diode-array detector (DAD). The hydroperoxides were detected specifically at wavelength 234 nm, and the isomers were identified based on their maximum absorbance wavelength (Bisakowski et al. 2000; Perraud and Kermasha 2000). In addition, an LC-MS/MS method was shown to be useful for analysing oxidation products from PUFAs in wheat varieties (Levandi et al. 2009). In that study, complex oxidation compounds, including tri-, di- and mono-hydroxides, epoxides and oxo-hydroxy products, were separated

using LC and further identified using MS and electrospray ionisation (ESI). Furthermore, LC-MS was used to analyse the hydroperoxides from soybean LOX-derived fatty acids (Schneider et al. 1997). The method was verified as able to quickly separate and identify the fatty acid hydroperoxides without any derivatisations prior to the LC-MS analysis.

In summary, ASE methods have been shown to efficiently extract lipids from food matrices, but more studies are needed regarding how to properly extract NVOFA products. Both GC and LC methods are popularly used to analyse the NVOFAs, and these methods are able to quantify and identify the products to indicate lipid oxidation and study the compounds that may contribute to off-flavours, such as bitterness in foods.

3 AIMS OF THE STUDY

The overall aim of this thesis was to study the role of enzymatic and chemical lipid degradation reactions in forming off-flavour compounds from oat and faba bean matrices. The special focus was on investigating the activities and properties of lipase, LOX and POX as lipid-modifying enzymes in oat and faba bean.

The detailed objectives were:

1. To investigate the activities of lipase, LOX and POX present in oat and faba bean cultivars and to evaluate their contributions to the formation of compounds related to lipid-derived off-flavours. Furthermore, a method was developed to study POX activity based on specific production of epoxy compounds (Study I).

2. To study further the significance of POX activity in oat by investigating the occurrence of NVOFAs during storage of oat, as well as their relations to the release of FFAs and the formation of volatile products. An ultra-high performance liquid chromatography coupled with evaporative light scattering detection or MS (UHPLC-ELSD/MS) method was developed to analyse the occurrence and formation of NVOFAs (Study II).

3. To investigate further the role of lipid-modifying enzymes in faba bean in causing potential off-flavour compounds. The optimum pH and substrate specificity of lipase and LOX were characterised, as well as the product specificity of LOX and the LOX pathway to produce specific volatile lipid oxidation products from various substrates. In addition, the overall potential of faba bean lipid-modifying enzymes in forming volatile lipid oxidation products in food models was studied (Study III).

4 MATERIALS AND METHODS

This chapter summarises the materials and analytical methods used in the study. The purity and origins of reagents and more details of the experiments and analytical methods were presented in papers I–III.

4.1 Lipid-modifying enzymes in oat and faba bean (Study I)

In the first study of this thesis, the activities of lipase, LOX and POX were determined in native oat grains and faba beans from different cultivars and cultivation years. In addition, a method was developed to study POX activity based on the production of epoxy compounds from substrates with cumene hydroperoxide added as an oxidant. The epoxy products were determined using chromatographic methods.

4.1.1 Oat and faba bean samples

Grain samples of four oat cultivars, namely Akseli, Alku, Meeri and Steinar, were provided by Boreal Plant Breeding Ltd. (Jokioinen, Finland) from the cultivation years 2012, 2013 and 2014. The samples were stored at 10–15 ^{o}C and milled without dehulling using a Centrifugal Mill ZM200 (Retsch, Haan, Germany) fitted with a 0.5-mm sieve. The milled oat flours were transferred immediately to a freezer and stored at -20 ^{o}C until analysis.

Faba bean samples of four cultivars, namely Kontu, Alexia, Fatima and SSNS-1, were obtained from the Viikki Experimental Farm from three cultivation years. The samples from cultivation years 2011 and 2015 were stored at 5 ^{o}C, and those from year 2010 were stored at room temperature. The detailed information on cultivation and weather conditions was reported by Lizarazo et al. (2015). The beans were milled without dehulling and stored in the way described above.

Oat flour made from dehulled oat grains and obtained from the Raisio Group (Nokia, Finland) was used as an in-house reference to monitor the analytical level of the measurement of POX activity. A faba bean flour from the cultivar Kontu cultivated in year 2011 at the Viikki Experimental Farm was used as an in-house reference when measuring the lipase and LOX activities.

4.1.2 Outline of the study

Lipase and LOX activities were measured in the oat and faba bean cultivar samples using the same enzyme extract (*Section 4.4.1.1*). Both lipase and LOX activities were measured using a spectrophotometric method. For the lipase activity measurement, *p*-NPB (Sigma-Aldrich, St. Louis, MO, USA) was used as a substrate, and linoleic acid was used in the LOX activity measurement (*Sections 4.4.1.2* and *4.4.1.3*). Each sample was extracted in duplicate, and measurements of enzyme activity were made in triplicate from each extract. One in-house reference sample was included in each extraction batch.

To study the activity and substrate specificity of POX in oat and faba bean, triolein and both FFAs and fatty acid methyl esters at various unsaturation levels were used as substrates (Nu-Check Prep, Elysian, MN, USA). A GC-FID/MS method was used to measure POX activity by analysing the specific epoxidation products from triolein and fatty acid methyl esters, specifically, methyl oleate, methyl linoleate and methyl linolenate. In addition, a UHPLC-ELSD/MS method was developed to analyse the specific epoxy fatty acids oxidised from FFA substrates, including oleic, linoleic and linolenic acids. The detailed method development for epoxide analysis is shown in *Section 4.4.1.4*.

4.2 Occurrence and formation of epoxy and hydroxy fatty acids as NVOFAs in oat (Study II)

The second study of this thesis focused on investigating the occurrence and formation of the potential NVOFAs, epoxy and hydroxy fatty acids in oat. To reach this aim, the oat samples included both heat-treated (HT) and NHT ones, and the samples were studied both fresh and after storage.

4.2.1 Oat samples to study the occurrence of NVOFAs

To form NVOFAs, an NHT oat flour sample made from dehulled oat grains (Raisio Group, Nokia, Finland) was stored at 35 °C for 10 weeks. After storage, the sample was used as an in-house reference to verify the analytical level of the measurements and develop the method regarding NVOFAs.

The occurrence of NVOFAs was studied using three aged commercial oat flour samples made from dehulled oat grains, including an old HT oat flour stored in a freezer for several years (O-HT-F1), an old HT oat flake sample stored at room temperature for more than one year (O-HT-

F2) and milled before analysis and an old NHT oat powder stored at room temperature for one year (O-NHT-P). In addition, to observe the occurrence of NVOFAs, two other flour samples from NHT dehulled oat grains were included: freshly milled NHT oat flour (F-NHT-F1) and freshly purchased NHT oat flour (F-NHT-F2). The oat grains for F-NHT-F1 had been stored at 4 °C for several years and were milled in the way described in *Section 4.1.1*. To investigate the formation of NVOFAs in oat, a storage experiment was conducted using the freshly milled F-NHT-F1 and F-NHT-F2 samples, which were stored at 35 °C for 6, 12, 18 and 24 weeks. The freshly milled samples (0 week) were used as an initial point.

4.2.2 Oat samples for controlled storage experiment

To further study the effect of heat treatment on the formation of NVOFAs during oat storage, a controlled experiment was conducted to measure formation of NVOFAs in the flours of NHT and HT dehulled oat grains from two cultivars (NHT/HT-CV1 and CV2) (Raisio Group, Raisio, Finland), as well as in industrial-scale NHT and HT dehulled oat grain samples (NHT/HT-IS) (Fazer Mills, Vantaa, Finland), which were milled using the method described in *Section 4.1.1*. Then, the milled flour samples were divided and subjected to a storage experiment, as shown in Table 1. Briefly, samples were divided into different clean glass bottles (*ca.* 10 g each) for each storage time point, the caps were tightly closed and the samples were stored at 40 °C for one, two, three or four months. The freshly milled samples (0 month) were taken as the initial points (Table 1).

Table 1. Summary of the analysis of non-volatile oxidised fatty acids (NVOFAs), lipid hydrolysis and volatiles of oat samples stored at 40 °C *.

Oat samples	Analysis	0 month	1 month	2 month	3 month	4 month
NHT-CV1 (the flour made from non-heat treated oat grains-cultivar 1)	NVOFAs	v	v	v	v	v
	Lipid hydrolysis	v	v	-	v	v
	Volatiles	v	v	-	v	-
HT-CV1 (the flour made from heat treated oat grains-cultivar 1)	NVOFAs	v	v	v	v	v
	Lipid hydrolysis	v	v	-	v	v
	Volatiles	v	v	-	v	-
NHT-CV2 (the flour made from non-heat treated oat grains-cultivar 2)	NVOFAs	v	v	v	v	v
	Lipid hydrolysis	v	v	-	v	v
	Volatiles	v	v	-	v	-
HT-CV2 (the flour made from heat treated oat grains-cultivar 2)	NVOFAs	v	v	v	v	v
	Lipid hydrolysis	v	v	-	v	v
	Volatiles	v	v	-	v	-
NHT-IS (the flour made from industrial-scale non-heat treated oat grains)	NVOFAs	v	v	v	v	v
	Lipid hydrolysis	v	v	v	-	v
	Volatiles	v	v	v	-	v
HT-IS (the flour made from industrial-scale heat treated oat grains)	NVOFAs	v	v	v	v	v
	Lipid hydrolysis	v	v	v	-	v
	Volatiles	v	v	v	-	v

* v : measured; - : not measured.

4.2.3 Outline of the analyses

To analyse the NVOFAs, a new method was developed and validated that included ASE extraction, solid phase extraction (SPE) fractionation of the lipid extract and use of the UHPLC-ELSD/MS method for identification and quantification (*Section 4.4.2*). To relate the formation of NVOFAs to the formation of FFAs and volatile lipid oxidation products, TAGs, FFAs and volatile oxidation products were analysed in selected oat samples at the time points shown in Table 1. TAGs and FFAs were extracted using an ASE method, and their contents were analysed using NP-HPLC-ELSD (*Section 4.4.3*). Volatile oxidation products were analysed using HS-SPME-GC-MS (*Section 4.4.4*). For volatile analysis, 1-g samples were weighed directly into 20-ml dark brown vials, closed tightly and stored at 40 ^{o}C. In addition, the performance of the HS-SPME-GC-MS was monitored using a reference sample that was milled from a porridge mixture of oat flakes and raspberries (Elovena, Raisio Group, Raisio, Finland). The analysis of oat samples was done in triplicate for each storage time point.

4.3 Potential of faba bean lipid-modifying enzymes to promote formation of volatile lipid oxidation products (Study III)

In Study III, faba beans of different cultivation years were used to characterise the pH optima and substrate specificity of lipase and LOX, as well as the product specificity of LOX and the LOX pathway to produce specific volatile lipid oxidation products from various substrates. In addition, to study the formation of volatile lipid oxidation products catalysed by the lipid-modifying enzymes of faba bean, emulsions made from rapeseed oil (RO) or rapeseed oil fatty acids (ROFA) containing faba bean extract, as well as the extract without added oil, were used as food models.

4.3.1 Faba bean samples

Faba bean samples of the Kontu cultivar from the cultivation years 2011, 2015 and 2016 were obtained from the Viikki Experimental Farm. The samples were milled without dehulling and stored at -20 ^{o}C in the way described in *Section 4.1.1*. A faba bean sample from 2016 was used to measure the optimum pH of lipase, while another sample from 2011 was used to study the optimum pH of LOX and the volatile profiles formed from linoleic and linolenic fatty acids. In addition, samples from 2015 and 2016 were used to characterise the activities and properties of lipase and LOX in faba bean and to study the formation of volatile lipid oxidation products in food models under selected pH conditions.

4.3.2 Outline of the study

To characterise faba bean lipase, its activity was determined at pHs 4.5, 6, 7, 7.5 and 8 using *p*-NPB as a substrate (method in *Section 4.4.1.2*). The substrate specificity of faba bean lipase was studied using substrates with various sizes of acyl moiety, *p*-NPB and *p*-NPP (Sigma-Aldrich, St. Louis, MO, USA) (method in *Section 4.4.1.2*) and with pure TAGs (triolein and trilinolein, > 99%, Nu-Check Prep, Elysian, MN, USA) and RO (Raisio, Bunge, Finland). For comparison, the lipase activity in an oat sample (cultivar Steinar, 2014) was measured. Finally, the NP-HPLC-ELSD method was used to measure the hydrolysis efficiency of the TAGs (*Section 4.4.3*).

To characterise faba bean LOX, its activity was compared at pHs 4, 5, 6, 6.8 and 8.5 using linoleic acid as a substrate (method in *Section 4.4.1.3*). To characterise the product specificity of LOX, faba bean extracts were incubated with linoleic acid to produce 9- and 13-hydroperoxides, and the products were analysed as described in *Section 4.4.1.3*. To characterise the substrate specificity of the LOX pathway in producing volatile lipid oxidation compounds, the formation of volatile lipid oxidation products was evaluated by comparing linoleic acid, linolenic acid and trilinolein as substrates. Faba bean extracts were incubated with 10 mM of linoleic/linolenic/trilinolein substrate solution at pH 6. The reaction was begun by adding 2.2 ml of the 0.1 M potassium phosphate buffer (pH 6.0) to 20-ml amber SPME vials and then adding 400 µl of the substrate solution and 400 µl of the faba bean extract, after which the vials were closed with aluminium caps with PTFE/silicone septa. The bottles were incubated in a water bath at 30 °C for 10 min, and the reaction was stopped by adding 300 µl of 1 M HCl through the cap septa. With the control samples, to indicate chemical lipid oxidation, the HCl was added before the faba bean extract was added. Volatile lipid oxidation products were analysed using HS-SPME-GC-MS (*Section 4.4.4*). All measurements were made on duplicate extracts with two replicate incubations and with one control from each extract.

Finally, four models were used to study the overall role played by lipid-modifying enzymes in faba bean in the formation of volatile lipid oxidation products. The faba bean extract was studied as such and as 3% and 5% emulsions with RO and ROFA under three pH conditions: pHs 5, 6.4 and 8. A faba bean flour sample of 3 g was extracted with either 12 ml of Milli-Q-H_2O (pH of the extract was 6.4) or 0.1 M of KH_2PO_4 buffer (pHs 5 and 8). The mixture was vortexed and let stand at room temperature for 10 min, after which the pH was re-adjusted for the samples extracted at pHs 5 and 8, the slurry was centrifuged at 10000 g for 10 min, and the

supernatant was collected. To prepare the emulsions, faba bean extract was mixed with either RO or ROFA using an Ultra-Turrax (IKA®-Werke GmbH & Co. KG, Germany) at 8000 rpm for 2 min. The extract and the emulsions were divided in triplicates of 3 ml each and then subjected to the HS-SPME-GC-MS (*Section 4.4.4*). Each measurement was made from a single extract in triplicate.

4.4 Analytical methods

4.4.1 Measuring activity of lipid-modifying enzymes in oat and faba bean

4.4.1.1 Enzyme extraction (Studies I & III)

In Study I, the lipid-modifying enzymes were extracted by mixing 2.5 g of oat/faba bean flour with 23 ml of 0.1 M potassium phosphate buffer (pH 6.7). The mixture was vortexed (4 times × 10 s) and kept on ice for 1 hr. Then, the slurry was centrifuged at 9000 × g for 10 min at 4 ºC (Sorvall RC5C, SLA-1500 rotor), and the supernatant was collected and centrifuged for another 10 min. Finally, the supernatant was kept on ice until the enzyme activity was analysed.

In Study III, to characterise the lipase and LOX from faba bean flours, the enzymes were extracted differently. To measure lipase activity using *p*-NPB and *p*-NPP as substrates, 2 g of faba bean flour was stirred with 10.0 mL of MilliQ-water and kept on ice for 1 h. To characterise the faba bean lipase using TAGs and RO, 4 g of faba bean flour was stirred with 10.0 mL of MilliQ-water and kept on ice for 1 h. The mixture was centrifuged at 9600 × g at 4 °C for 15 min, after which the supernatant was collected and kept on ice until analysis. To characterise the faba bean LOX, more diluted extract was prepared, i.e. 0.5 g of faba bean flour was mixed with 20 ml of MilliQ-water and stirred 4 times × 10 s, after which the slurry was kept at room temperature for 15 min and then centrifuged at 10,000 × g for 15 min. The supernatant was collected and kept on ice until analysis.

4.4.1.2 Measuring lipase activity (Studies I & III)

Measuring lipase activity using p-NPB

Lipase activity was measured using *p*-NPB as a substrate and a spectrophotometric method (Brunschwiler et al. 2013). The *p*-NPB substrate solution was prepared by dissolving an aliquot of 50 mM of stock solution into KH_2PO_4 buffer (+ 0.1% Triton X-100) at pH 8 to a final concentration of 2 mM, and the emulsion was made by vortexing 4 times × 10 s. In the measurement, either 20 µl of faba bean extract or 80 µl of oat extract was mixed with the *p*-NPB substrate solution to a total volume of 1 ml, and the increase of the absorption during 150

s was collected using a spectrophotometer at 405 nm (Lambda 25 UV/Vis, Perkin Elmer Inc., USA). This method was also used to measure lipase activity in oat and faba bean at pH 8 in Study I. The molar extinction coefficient value of 16.05 mM^{-1} cm^{-1} for *p*-nitrophenol was used to calculate the results. Finally, the lipase activity was expressed as μmol min^{-1} g^{-1} flour. Each measurement was made from two extracts with triplicate lipase activity measurements (n = 6). In Study III, a 2 mM *p*-NPB substrate solution was made by diluting the stock solution with 50 mM of KH_2PO_4 buffers at pHs 4.5, 6, 7, 7.5 and 8 to study the effect of pH on lipase activity.

Measuring substrate specificity of faba bean lipase (Study III)

To examine the effect of the size of the acyl moiety on lipase activity, the *p*-NPP substrate was also used. Its preparation was similar to that described above, except that the emulsion was made by mixing the substrate solution using an Ultra-Turrax® T25 homogeniser at 8000 rpm for 5 min and that the solution was kept on ice during homogenisation. The reaction was conducted by mixing 3.6 ml of the emulsion and 0.4 ml of enzyme extract in a total volume of 4 ml. The 4-ml mixture was evenly divided into two parts: The first was filtered through a 0.45-μm filter and immediately measured with a spectrophotometer to obtain the initial absorption value, and the second was incubated in a shaking water bath at 37 °C for 30 min and then filtered and measured. The results were given as μmol min^{-1} g^{-1} flour. Each measurement was made from two extracts in triplicate (n = 6).

To study the activity of faba bean lipase using TAGs (triolein, trilinolein) and RO as substrates, 200 mg of each substrate was mixed with 15 ml of buffer (0.05 M Tris-HCl buffer at pHs 7.5 and 8, 1.8% Triton X-100) using an Ultra-Turrax® T25 homogeniser to make an emulsion. To begin the enzymatic reaction, 0.5 mL of the enzyme extract was mixed with 1.5 ml of emulsion in a tube, and then the mixture was incubated at 37 °C in a shaking water bath for 1.5 h. The reaction was stopped by adding 3 mL of ethanol and 100 μl of 1 M HCl. Finally, the lipids were extracted twice with 10 ml of diethyl ether, and the solvent was evaporated at 35 °C using rotary evaporation. The dried residue was immediately re-dissolved in 10 mL of heptane, 1 ml of which was further dissolved into 10 ml of heptane. Then, the solution was filtered through a 0.45-μm film, and 10 μl of the sample was injected and analysed using NP-HPLC-ELSD, as described in *Section 4.4.3*. Each flour sample was extracted in duplicate, and each extract was measured in triplicate (n = 6).

4.4.1.3 Measuring LOX activity and hydroperoxide isomers produced by faba bean LOX (Studies I & III)

Measuring LOX activity

LOX activity was measured using linoleic acid as a substrate (Jiang et al. 2016). Briefly, the reaction was initiated by mixing 200 µl of 10 mM linoleic acid substrate solution and 200 µl of sample extract into 2.6 ml of 0.1 M KH_2PO_4 buffer (pH 6) at 30 °C in a water bath for 3 min. Then, 3 ml of 0.1 N KOH solution was added to stop the reaction. For blank samples, the 0.1 N KOH solution was added into the buffer solution before adding the substrate solution and extract. The formation of conjugated hydroperoxides by oat or faba bean LOX from linoleic acid was detected at 234 nm using a spectrophotometer (Lambda 25 UV/Vis, Perkin Elmer Inc., USA). The results were calculated based on the molar absorptivity of conjugated dienes: ε = 26.000 l/mol cm. The LOX activity was given as mmol min^{-1} g^{-1} flour. Each flour sample was extracted in duplicate, and each extract was measured in triplicate (n = 6).

Measuring hydroperoxide isomers (Study III)

Hydroperoxides were produced by adding 400 µl of 10 mM linoleic acid substrate solution and 400 µl of faba bean enzyme extract into 2.2 ml of potassium phosphate buffer at pH 6. The hydroperoxide isomers were identified based on those produced from linoleic acid by soybean LOX 1-B (lyophilised powder, ≥ 50,000 units/mg solid, Sigma-Aldrich, UK) that demonstrated activity of 20,000 U/ml. This enzyme is known to produce mainly 13-HPOD (Gardner 2003). The mixture was incubated in a water bath at 30 ºC for 10 min, and then the reaction was stopped by adding 150 µl of 1 M HCl. Blank samples were made by adding 150 µl of 1 M HCl before adding the enzyme extract. The hydroperoxides were extracted using 10 ml of diethyl ether twice, and the extract was washed with 5 ml of saturated NaCl solution. Finally, the solvent was evaporated and the residue dissolved into 10 ml of heptane solution. An aliquot of 80 µl of the solution was injected and analysed using an NP-HPLC-DAD set at λ= 234 nm. The same NP-HPLC method was used to analyse TAGs (*Section 4.4.3*), except that DAD was used instead of ELSD.

4.4.1.4 Developing method for measuring POX activity using GC-FID/MS and UHPLC-ELSD/MS (Study I)

Developing the GC-FID/MS method

To measure POX activity, methyl oleate, methyl linoleate and methyl linolenate were used as methyl ester substrates. The substrates were incubated with cumene hydroperoxide and faba

bean and oat extracts, and the epoxy fatty acid methyl esters formed were measured using GC-FID/MS.

About 1.3 mg of each fatty acid methyl ester was mixed with internal standard methyl nonadecanoate (me-C19:0; Nu-Check Prep, Elysian, MN, USA) in heptane. The mixture was evaporated using N_2, and the dried residue was mixed with 350 µl of Milli-Q water solution containing 1% Tween 20 by vortexing. Then, 1.75 ml of HEPES solution (100 mM, pH 7.0), 900 µl of Milli-Q water and 500 µl of the enzyme extract were added to a final volume of 3.5 ml. Next, 7 µl of 8% cumene hydroperoxide diluted in ethanol solution was added to begin the reaction, and the mixture was incubated in tubes inverted by a rotator for 1 h at room temperature. Finally, the reaction was ended by adding 5 ml of methanol, and the compounds were extracted using 10 ml of diethyl ether twice. The extract was collected and evaporated using N_2, and the dried residue was dissolved into 1 ml of heptane. For the reaction of triolein, the assay was done in the same way as described above, except that the final residues were dissolved into 1.5 ml of heptane, and the substrates and products were methylated using an alkaline transesterification method (Christie 1993). Finally, the analytes were subjected to GC-FID/MS analysis. The levels of epoxidation products and substrate residues were calculated as mg based on an internal standard method, and it was assumed that all analytes gave equal FID responses. The final results were given as the proportion (%) of epoxides formed from the amount of original substrate, and the proportion of substrates left (%) was also given. Each flour sample was extracted in duplicate, and each extract was measured in triplicate (n = 6).

The substrates and their epoxide products were also analysed using GC-FID/MS. A GC-FID (Agilent 6890N, USA) coupled with a silica capillary column (Omegawax™ 250, 30 m × 0.25 mm × 0.25 µm, SUPELCO®, Bellefonte, USA) was used to quantify the compounds, and a GC (HP 6890 series, Agilent Technologies Inc., Wilmington, DE, USA) equipped with an MS detector (Agilent 5973 Network, Agilent Technologies Inc., Wilmington, DE, USA) and the same capillary column were used to identify the epoxide.

Methyl oleate and methyl linoleate were used as substrates to test the repeatability and validation of the method. In each set of analyses, an in-house oat reference sample was used to monitor the analytical level and to validate the method using methyl oleate as a substrate.

Developing the UHPLC-ELSD/MS method

To study POX activity toward FFA at various levels of unsaturation, a UHPLC-ELSD/MS method was developed to analyse the epoxides from FFAs at various unsaturation levels: oleic, linoleic and linolenic acids. A working range test for each FFA substrate and internal standard (C19:0) was performed in a range from 30–2100 ng/injection. To test the performance of the final method, *ca.* 1.5 mg of each substrate and 420 µg of the internal standard were incubated with oat extract to form the epoxy fatty acids. The enzyme reaction process was the same as noted above in the fatty acid methyl esters analysis. After evaporation, the residues were immediately dissolved into 1 ml of isopropanol and 1 ml of methanol.

A UHPLC-ELSD (Waters Acquity, Milford, MA, USA) with a reversed-phase Acquity UHPLC® HSS T3 column (1.8 µm, 2.1 × 150 mm, Waters, Ireland) was used to quantify the epoxides and FFAs. Nonadecanoic acid (3.16 mg/ml, Nu-Check Prep, Elysian, MN, USA) was used as an internal standard and mixed with each substrate for analysis. The separation of the compounds was conducted in a 15-min gradient elution program consisting of Milli-Q-water with 0.05% acetic acid (as eluent A) and methanol with 0.05% acetic acid (as eluent B) at a flow rate of 0.3 ml/min at a column temperature of 30 °C. The contents of epoxides were calculated based on the second-order standard curves made from the internal standard nonadecanoic acid and the 9,10-epoxystearic acid standard (Santa Cruz Biotechnology®, Texas, USA). The levels of epoxidation products and substrate residues were calculated as mg based on the standard curves, and the final results were expressed as the proportions (%) of the epoxides formed from the original substrates. The proportions of the substrates left (%) were also given. Each flour sample was extracted in duplicate, and each extract was measured in triplicate (n = 6).

The epoxides identified from the FFAs using a UHPLC-quadrupole-time-of-flight (UHPLC-Q-TOF) (Acquity I class, Milford, MA, USA) in a negative mode and having an electrospray ionisation (ESI) interface, coupled with a SYNAPT G2-Si mass spectrometer. The elution was conducted using the same column and gradient used in the UHPLC-ELSD analysis described above. Briefly, the settings for the instrument were as follows: *m/s* scan range, 50–1200; capillary voltage, 2.5 kV; source offset, 80 V; source temperature, 100 °C; sampling cone, 40 V; desolvation temperature, 500 °C; gas flow, 1000 l/h; cone gas flow, 100 l/h; nebuliser gas flow, 6.2 bar; ramp for MS/MS trap collision, 10–70 eV; and scan time, 0.3 s.

In each batch of sample analysis, an in-house reference sample of oat that had oleic acid as a substrate was used to monitor the analytical level of the measurements. In addition, this in-house oat reference was used as an indicator of the relative stability of the POX activity in the oat samples that were stored in the freezer and measured in different batches.

4.4.2 Developing method of analysing NVOFAs in oat (Study II)

4.4.2.1 Lipid extraction using ASE

A gentle method (Fig. 7) of extracting the lipids from oat samples using ASE (Dionex ASE-200, Dionex Corporation, Sunnyvale, CA, USA) was developed based on the study of Yao and Schaich (2014). From that study, the temperature of 40 ^{o}C and the use of two cycles for lipid extraction were adopted, but the present study evaluated different extraction solvents and selected the solvent that gave the highest yield of total lipids. The solvents included acetone, heptane:ethanol (2:1, vol%:vol%) and ethanol. The sample preparation was conducted as reported by Lampi et al. (2015). About 1 g of oat flour was mixed with an equal amount of Ottawa sand in an 11-ml extraction cell, and then the cell was filled to the top with the same sand. The lipid extraction parameters were as follows: pressure, 1500 psi; cell temperature, 40 ^{o}C and cell preheating period, 5 min. Two static extraction cycles of 20 min were used. After lipid extraction and solvent evaporation, the dried residue was re-dissolved using dichloromethane (DCM):heptane (1:9, vol%:vol%) into a final volume of 2 ml prior to the removal of TAGs.

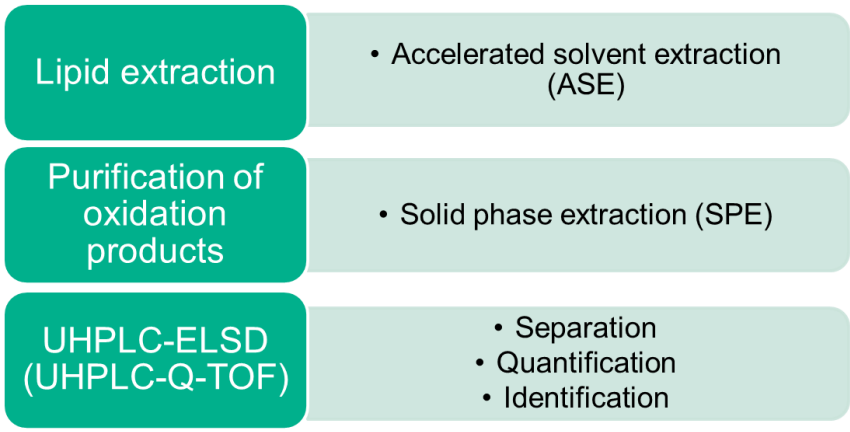


Fig. 7. Extraction, purification and analysis of NVOFAs from oat flour samples.

4.4.2.2 Developing method of fractionating lipids using SPE

Several tests were conducted to study the recovery of NVOFAs in SPE fractionation. For instance, the lipids retained in the SPE cartridge were first washed with 10 ml of the initial eluent containing heptane:diethyl ether (9:1, vol%:vol%) to remove the TAGs, followed by elution of the NVOFAs with heptane:diethyl ether (vol%:vol%) containing 1% acetic acid in ratios of 70:30, 50:50, 20:80 and 0:100 sequentially, using 5 ml of each of them. After testing at several volumes and solvent ratios, a lipid fractionation procedure was selected for purifying and collecting the NVOFAs by SPE, as shown in Fig. 8. Briefly, the silica cartridge (Strata SI-1 Silica, 55 µm, 70 A, 500 mg/3ml) was activated by adding 2 × 2.5 ml of heptane, and then 1 ml of the sample extract obtained in *Section 4.4.2.1* was added. Next, 4 × 2.5-ml of heptane:diethyl ether (90:10, vol%:vol%) solution was used to remove neutral lipids, including TAGs and the majority of FFAs. Finally, NVOFAs were eluted using heptane:diethyl ether (20:80, vol%:vol%) containing 1% acetic acid from the cartridge. The collected solution was evaporated using nitrogen at 35 ^{o}C, and the dried residue was re-dissolved into 0.5 ml of 2-propanol and 0.5 ml of acetonitrile.

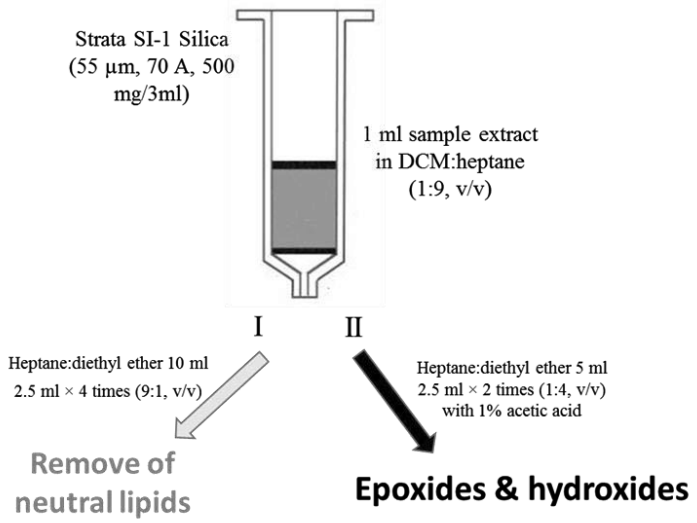


Fig. 8. Purification of lipid extracts to collect NVOFAs by SPE.

The 12-hydroxyoctadecanoic acid standard (Sigma-Aldrich, St. Louis, MO, USA), 12,13-epoxy-octadec-9-enoic acid and 9,10-epoxy-octadec-12-enoic acid were used to study the recoveries of epoxy and hydroxy fatty acids from ASE extraction and SPE fractionation. The 12,13-epoxy-octadec-9-enoic acid and the 9,10-epoxy-octadec-12-enoic acid were produced by oat POX using a method described in *Section 4.4.1.4*. Finally, to test the recovery of oxidised

fatty acids, *ca.* 2 mg of epoxides and 1 mg of 12-hydroxyoctadecanoic acid in 1 ml of ethanol were transferred to ASE cells and mixed with 1 g of oat flour. For blank samples, only 1 ml of ethanol was used. The recovery test was conducted using the steps shown in Fig. 7.

4.4.2.3 NVOFA analysis using UHPLC-ELSD/MS

A UHPLC-ELSD/MS method was developed to analyse NVOFAs. Linoleic and linolenic acids were subjected to autoxidation at 40 ^{o}C to form hydroperoxides, which were further converted into epoxy and (epoxy-)hydroxy fatty acids using incubation with oat extracts. The compounds were extracted using solvents, mixed with the 12-hydroxyoctadecanoic acid standard, dried with N_2 and dissolved into 2-propanol and acetonitrile for testing using the UHPLC-ELSD/MS method.

The UHPLC-ELSD instrument was the same as described in *Section 4.4.1.4*, except that the eluents consisted of Milli-Q water with 0.05% acetic acid as solvent A and acetonitrile with 0.05% acetic acid as solvent B. The elution was done using a column temperature of 30 ^{o}C, a flow rate of 0.3 ml/min and a 28-min gradient elution. The injection volume was 4 µl of solution for both the UHPLC-ELSD and the UHPLC-Q-TOF analyses. To quantity the oxidised fatty acid products, the 9,10-epoxyoctadecanoic and the 12-hydroxyoctadecanoic acid standards were used. The amounts of the hydroxy and epoxy-hydroxy products were calculated based on the second-order standard curves made using 12-hydroxyoctadecanoic acid (range from 80–2000 ng/injection), while the epoxides were calculated using the second-order standard curves of the 9,10-epoxyoctadecanoic acid (range from 80–2000 ng/injection). The results were provided as µg/g of oat flour. Each oat sample was extracted and analysed in triplicate. The NVOFAs identification was conducted using UHPLC-Q-TOF and the same column and programme used for the UHPLC-ELSD. The setup used was the same as used for MS in *Section 4.4.1.4*.

Furthermore, the performance of the method developed was evaluated using the recoveries of the compounds tested: 12-hydroxyoctadecanoic acid standard, 12,13-epoxy-octadec-9-enoic acid and 9,10-epoxy-octadec-12-enoic acid.

4.4.3 Analysing neutral lipids

Lipid hydrolysis was studied by measuring neutral lipids in oat samples, mainly TAGs and FFAs. The lipids were extracted using an ASE method described by Lampi et al. (2015). An

NP-HPLC (Agilent 1200HPLC system, Agilent Technologies, Santa Clara, CA, USA) coupled with ELSD (Waters 2420 ELSD, Waters®, Milford, MA, USA) and a LiChrosorb diol column (5 mm, 3 × 100 mm, VDS Optilab Chromatographie Technik GmbH, Berlin, Germany) was used to analyse the contents of the neutral lipids (Lampi et al. 2015). The lipids were separated in a gradient of 60 min with heptane and 0.1% acetic acid as solvent A and a mixture of heptane and 3% 2-propanol containing 0.1% acetic acid as solvent B at a flow rate of 0.5 ml/min and a column temperature of 25 °C. The lipid classes were quantified using an external standard method, and the second-order of the standard curves for TAG and FFA analysis were made from a mixture of the triglyceride palmitate and oleic acid standards (100–5000 ng/injection).

4.4.4 Analysing volatile profiles from lipid oxidation (Studies II & III)

The HS-SPME-GC-MS method (Damerau et al. 2014) was used to study the formation of volatile lipid oxidation products in oat during storage (Study II) and the formation of volatile lipid oxidation products by faba bean lipid-modifying enzymes in food models (Study III). The volatile compounds were extracted using a DVB/CAR/PDMS-fibre (50/30 µm) at 60 °C for 30 min, after which they were separated using GC and an SPB-624 capillary column (30 m × 0.25 mm i.d., 1.4 mm) and further identified and quantified using MS and their total ion chromatograms (*m/z* from 40–300). The results for volatiles were given as peak areas (counts * s * 10^6).

4.5 Data analysis

Statistical analysis was conducted using SPSS (IBM SPSS Statistics, USA) to study the effects of cultivars and cultivation years and the interactions between the two on the activities of lipid-modifying enzymes. A value of $p < 0.05$ was considered statistically significant. The results were analysed using two-way analysis of variance (ANOVA), and Tukey tests were used to identify sample homogenous groups (Study I). All results were given as mean values ± standard deviation for different replicates of samples. The figures were created using OriginPro 8.6 (OriginLab Corporation, USA).

5 RESULTS

The detailed results of studies I–III are shown in papers I–III.

5.1 Developing and evaluating methods of analysing POX products

5.1.1 Determining of the epoxides to measure POX activity

POX activity in oat and faba bean was measured based on the amount of epoxides formed from FFAs, fatty acid methyl esters and triolein incubated with enzyme extract in the presence of cumene hydroperoxide as an oxygen donor. The incubations were conducted at pH 7 for 1 h at room temperature, and only epoxides were selectively produced. In addition, we were able to learn more about the substrate specificity of POX and to provide a reliable method of measuring POX activity. The epoxy products of fatty acid methyl ester and triolein were measured using GC-FID/MS, and to analyse epoxides from FFA substrates, a UHPLC-ELSD/MS method was developed.

5.1.1.1 Epoxides from fatty acid methyl esters using GC-FID/MS (Study I)

Epoxides of methyl oleate, linoleate and linolenate and their corresponding fatty acid methyl esters were separated using the GC-FID method, as shown in Fig. 9. Relative retention times (R_r: $t_{epoxide}/t_{me\text{-}C19:0}$) and major fragments ($m/z > 120$) obtained using the GC-MS method were used to identify the epoxides (Table 2). The R_r values of epoxides varied from 1.332–1.423 (Table 2), while the intact fatty acid methyl esters were eluted earlier than the epoxides (Fig. 9). The epoxide products were clearly separated from the earlier eluted fatty acid methyl ester substrates, and the internal standard, methyl nonadecanoic acid, was eluted between methyl linoleate and methyl linolenate.

During ionisation, the epoxides of fatty acid methyl esters decomposed into numerous small fragments, while the molecular ions (M^+) could hardly be detected (Table 2). The only epoxide formed from methyl oleate showed two major fragments: *m/z* 155 [M − 157, loss $\bullet(CH_2)_7COOCH_3$] and 199 [M − 113, loss $\bullet(CH_2)_7CH_3$] (Table 2). The product was identified as methyl 9,10-epoxyoctadecanoate. Two epoxides were formed from methyl linoleate (peaks 6 and 7, Fig. 9). The first eluted epoxide (peak 6) produced three characteristic fragments *m/z* 279 [M − 31, loss $\bullet OCH_3$], 207 [M – 103, loss $\bullet(CH_2)_5CH_3$ and H_2O] and 164 [M − 146, loss C_7OH_{15} and $\bullet OCH_3$]. The second one, (peak 7) produced fragments *m/z* 279 [M − 31, loss $\bullet OCH_3$], 185 [M − 125, loss $\bullet CH_3(CH_2)_4CH{=}CHCH_2CH_2$] and 168 [$M$ − 142, loss $\bullet OCH_3$ and C_8H_{15}]. They were identified as methyl 12,13-epoxy-octadec-9-enoate and methyl 9,10-epoxy-

octadec-12-enoate, respectively. However, the epoxides produced from methyl linolenate were co-eluted, which created a broad GC peak that produced many fragments (peak 8, Fig. 9), including *m/z* 277, 236, 207 and 155. All compounds obtained from epoxidation of fatty acid methyl esters that were catalysed by oat POX were mono-epoxy fatty acid methyl esters.

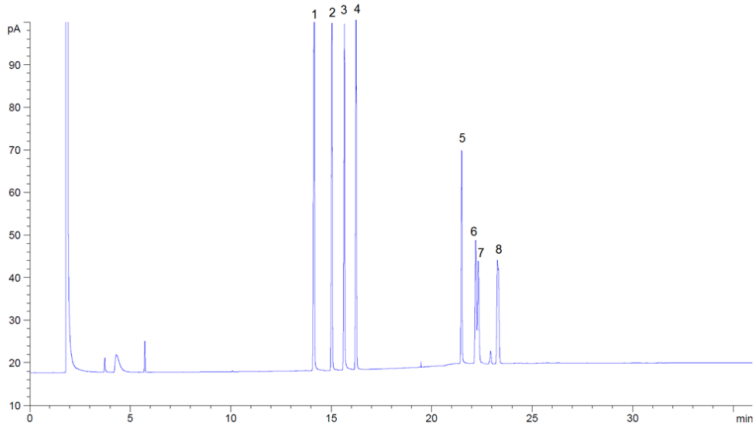


Fig. 9. GC-FID chromatogram of methyl oleate, methyl linoleate and methyl linolenate and their epoxidation products after incubation with oat POX. Where, 1: methyl oleate, 2: methyl linoleate, 3: methyl nonadecanoic acid (internal standard), 4: methyl linolenate, 5: methyl 9,10-epoxyoctadecanoate, 6: methyl 12,13-epoxy-octadec-9-enoate, 7: methyl 9,10-epoxy-octadec-12-enoate and 8: mixture of epoxides produced from methyl linolenate.

Table 2. Identification of epoxides formed from fatty acid methyl esters [a] and FFAs [b] incubated with oat extract containing POX using GC-MS analysis for fatty acid methyl esters and UHPLC-Q-TOF analysis for FFAs.

Fatty acid methyl ester [a]	**R_T ($t_{epoxide}/t_{me\text{-}C19:0}$)**	**Molecular ion M^+**	**Epoxide group(s)**	**Characteristic fragments (m/z > 120)**	**Mono-epoxidation product**
Methyl oleate	1.332	nd	1	155, 199	(9,10)
Methyl linoleate	1.354	nd	1	164, 207, 279	(12,13)
	1.361	nd	1	155, 168, 185, 279	(9,10)
Methyl linolenate	1.423	nd	1	121, 155, 207, 236, 277	mixture
FFA substrates [b]	**R_T ($t_{epoxide}/t_{C19:0}$)**	**Precursor ion $[M\text{-}H]^-$**	**Epoxide group(s)**	**Characteristic fragments (m/z > 150)**	**Mono-epoxidation product**
Oleic acid	0.649	297.2	1	171.1, 279.2	(9,10)
linoleic acid	0.575	295.2	1	183.1, 195.1, 277.2	(12,13)
	0.581	295.2	1	171.1, 277.2	(9,10)
Linolenic acid	0.532	293.2	1	171.1, 275.2	(9,10)
	0.537	293.2	1	223.1, 235.1	(15,16)
	0.549	293.2	1	195.1, 211.1, 275.2	(12,13)

Where, nd: not detected.

The epoxides, identified based on the R_r values obtained using GC-MS, were quantified using GC-FID analysis and methyl nonadecanoic acid as an internal standard. Analysis of the in-house oat reference sample in each set of samples using methyl oleate as a substrate yielded highly repeatable results. During the 1-h incubation, 14.2 ± 1.2% (n = 18) of the original substrate was oxidised into methyl 9,10-epoxyoctadecanoate. Thus, the method developed measured the epoxy products and the POX activity with good reliability and repeatability.

5.1.1.2 Epoxides from FFAs by UHPLC-ELSD/MS (Study I)

All the FFAs, oleic, linoleic and linolenic acids, and their epoxidation products as well as the internal standard, nonadecanoic acid, were separated during 15 min of elution time using the reversed phase (RP-)UHPLC-ELSD method, except for the products from linolenic acid, which were co-eluted (peak 1, Fig. 10). This method produced a wide working range of from 30–2100 ng for each fatty acid and showed good separation of and repeatable values for the FFA substrates and their epoxy products. The R_r ($t_{epoxide}/t_{C19:0}$) values of the epoxides obtained from UHPLC-Q-TOF ranged from 0.532–0.649, while the FFAs were eluted later than their epoxides (Table 2, Fig. 10). The epoxides were identified using UHPLC-Q-TOF and with ESI in a negative mode, which produced the deprotonated precursor ions $[M - H]^-$. The larger characteristic fragments of $m/z > 150$ obtained from each epoxide were structurally identified and their regio-isomers differentiated (Table 2).

Only one epoxide was formed from oleic acid, with a precursor ion of *m/z* 297.2 $[M - H]^-$, which was 16 units (an oxygen atom) larger than the precursor ion of oleic acid. Its characteristic fragments were *m/z* 155.1 and 171.1. Thus, the epoxide was identified as 9,10-epoxystearic acid (peak 4, Fig. 10). Two epoxides were formed from linoleic acid, and both yielded precursor ions of *m/z* 295.2 (Fig. 10 and Table 2). Among the fragment ions, *m/z* 183.1 and 195.1 were characteristic of the first epoxide (peak 2, Fig. 10), while *m/z* 171.1 was characteristic of the second one (peak 3, Fig. 10). Therefore, epoxide 1 was identified as 12,13-mono-epoxy-9-octadecenoic acid and epoxide 2 as 9,10-mono-epoxy-12-octadecenoic acid. A mixture of epoxide isomers produced from linolenic acid was obtained (peak 1, Fig. 10) with a single precursor ion, *m/z* 293.2, which was the deprotonated molecular ion of a mono-epoxide octadecadienoic acid (Table 2). These epoxide products could be identified by their typical fragments. One epoxide was elucidated based on the characteristic fragments *m/z* 171.1 and 183.0 as being 9,10-mono-epoxy-12,15-octadecadienoic acid. Another epoxide had characteristic fragment ions of *m/z* 223.2 and 235.2, and therefore was tentatively identified as

15,16-mono-epoxy-9,12-octadidecenoic acid. Finally, the last epoxide elucidated based on two characteristic negative ions, *m/z* 195.1 and 211.1, was identified as 12,13-mono-epoxy-9,15-octadidecenoic acid.

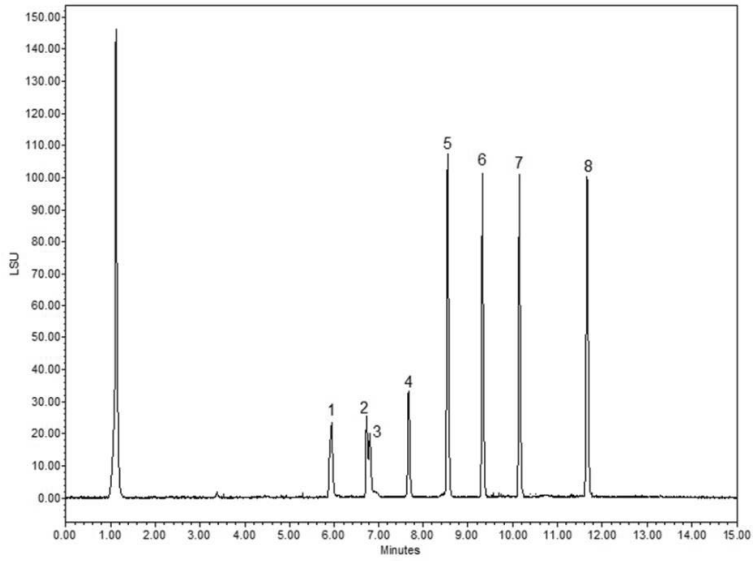


Fig. 10. UHPLC-ELSD chromatogram of oleic, linoleic and linolenic acids and their epoxidation products after incubation with oat POX. Where, 1: epoxides produced from linolenic acid, 2: 12,13-epoxy-9-octadecenoic acid, 3: 9,10-epoxy-12-octadecenoic acid, 4: 9,10-epoxystearic acid (a product of oleic acid as well as a standard), 5: linolenic acid, 6: linoleic acid, 7: oleic acid and 8: nonadecanoic acid (C19:0, internal standard).

Based on the R_r values obtained using the UHPLC-Q-TOF method, the epoxy fatty acids from FFA substrates were identified and further quantified using the UHPLC-ELSD method. The chromatogram also shows the separation of two standards, namely the internal standard nonadecanoic acid and the 9,10-epoxystearic acid standard, used to determine the second-order standard curves for quantifying epoxy products. In addition, the contents of each FFA and its residue after reaction were quantified using the second-order standard curves created from each FFA substrate and the internal standard. Finally, the proportion of the epoxidation products from the original substrate was used to express the POX activity. To follow the performance of the method, oleic acid was used as a substrate for POX, and a result of 54.9 ± 3.0% (n = 18) was obtained for production of 9,10-epoxystearic acid by POX from the in-house oat reference sample during a 1-h incubation. Therefore, the method was reliable and repeatable for analysing the epoxy fatty acids from FFAs and the POX activity in oat and faba bean.

5.1.2 Determining NVOFAs in oat flour (Study II)

5.1.2.1 ASE extraction and SPE fractionation

To extract NVOFAs, the ASE method was tested at 40 ºC with two extraction cycles using three solvent sets: acetone, heptane:ethanol (2:1, vol%:vol%) and ethanol. The heptane:ethanol (2:1) solvent gave the highest lipid yield from oat flour: 39.5 ± 0.9 mg/g flour as the sum of fatty acids (n = 6). Therefore, this solvent mixture was used to extract the NVOFAs. The removal of TAGs from the extract was achieved using SPE fractionation. After testing various ratios and volumes of solvent mixtures, all the TAGs and a large portion of the FFAs were removed using silica cartridge fractionation and heptane:diethyl ether (9:1, vol%:vol%) as an eluent, and the NVOFAs were fully eluted with 5 ml of heptane:diethyl ether (1:4, vol%:vol%) containing 1% acetic acid. The recovery values (n = 6) for the 12,13-epoxy-octadec-9-enoic acid and the 9,10-epoxy-octadec-12-enoic acid, which were added to the sample before extraction, were 89% and 90%, respectively, and for the 12-hydroxyoctadecanoic acid standard, the recovery value was 96% (n = 6).

5.1.2.2 Identifying and quantifying NVOFAs using UHPLC-Q-TOF and UHPLC-ELSD

The elution of the NVOFA compounds is shown in Fig. 11. To identify the epoxy and hydroxy compounds, relative retention times were calculated based on the retention time of the epoxy standard ($R_r = t_{compound}/t_{epoxy\ standard}$). Selected fragments were obtained using UHPLC-Q-TOF and ESI in a negative mode, which produced deprotonated precursor ions $[M-H]^-$. Fig. 12 (a–g) shows the corresponding spectra for the compounds C1-C7 obtained from Fig. 11, and the major characteristic fragments of *m/z* > 150 were selected for structural identification of its compounds. Each NVOFA compound had a fragment of *m/z* 18 less than the precursor ion, indicating that a molecule of H_2O was always lost during fragmentation, thereby forming the ion $[M-H-H_2O]^-$.

The earliest eluted NVOFAs (C1 and C2, Fig. 11.) possessed the largest molecular ions of all the compounds identified: *m/z* 313.2 and 311.2 (Fig. 12a, b, respectively). A fragment with high abundance at *m/z* 183.1 was obtained from C1, as shown in Fig. 12a, and high abundances of the fragments *m/z* 171.1, 199.1 and 211.1 were obtained from C2 (Fig. 12b). They were identified as 12,13-dihydroxy-octadec-9-enoic acid (C1, R_r = 0.301) and a mixture of 9-hydroxy-12,13-epoxy-octadec-10-enoic acid and 13-hydroxy-9,10-epoxy-octadec-11-enoic acid (C2, R_r = 0.347), respectively. The largest peak among all the NVOFAs was observed at an elution time of 9.2 min (C3 and C4, Fig. 11). Based on the spectra, two compounds were co-

eluted in this peak (Fig. 12c, d). Although both compounds had *m/z* 295.2 and 195.1, the abundance of $[M-H-H_2O]^-$ at 277.2 was the highest in Fig. 12d and much less in Fig. 12c. Another major difference was the fragment *m/z* 171.1, which could be seen in the spectrum in Fig. 12d, but was tiny in Fig. 12c. The compounds were identified as hydroxy regio-isomers: the first was 13-hydroxy-octadeca-9,11-dienoic acid (C3, $R_r = 0.521$, Fig. 11), and the second was 9-hydroxy-octadeca-10,12-dienoic acid (C4, $R_r = 0.526$, Fig. 11). Two other mono-epoxides were obtained from the oxidation of linoleic acid: 12,13-epoxy-octadec-9-enoic acid (C5, $R_r = 0.779$, Fig. 11) and 9,10-epoxy-octadec-12-enoic acid (C6, $R_r = 0.797$, Fig. 11). The last compound was 9,10-epoxystearic acid (C7, $R_r = 1.000$, Fig. 11) which was oxidised from oleic acid.

Based on the R_r, the identified NVOFAs in oat flours were quantified using UHPLC-ELSD. When the in-house oat reference sample in each batch of samples was analysed, repeatable values for the NVOFAs, 1964 ± 185 μg/g of flour, were obtained using the UHPLC-ELSD method (n = 9). Thus, the method developed was repeatable and reliable for analysing NVOFAs in oat.

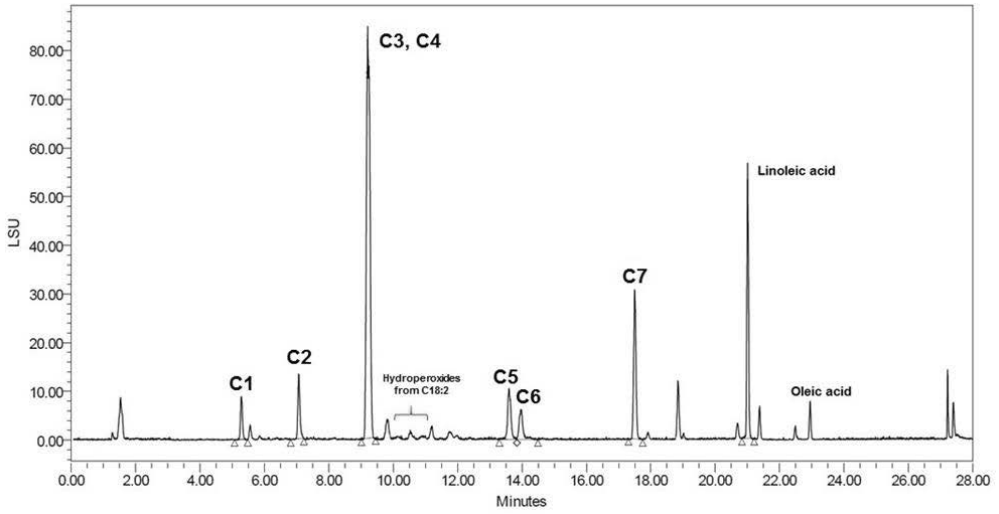
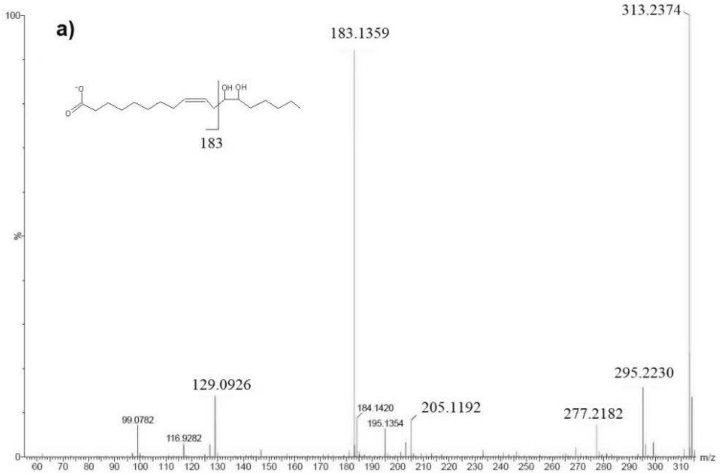


Fig. 11. UHPLC-ELSD chromatogram of oxidised fatty acids from oat lipid extracts after SPE fractionation. Where, C1: 12,13-dihydroxy-octadec-9-enoic acid; C2: a mixture of 13-hydroxy-9,10-epoxy-octadec-11-enoic acid and 9-hydroxy-12,13-epoxy-octadec-10-enoic acid; C3: 13-hydroxy-octadeca-9,11-dienoic acid; C4: 9-hydroxy-octadeca-10,12-dienoic acid; C5: 12,13-epoxy-octadec-9-enoic acid; C6: 9,10-epoxy-octadec-12-enoic acid and C7: 9,10-epoxyoctadecanoic acid.


a)
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183.1359
OH OH
183
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195.1354
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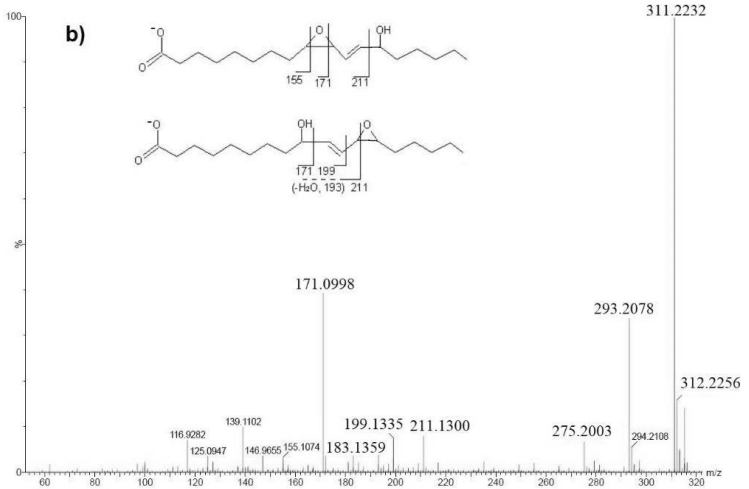
100
%
0
m/z


b)
311.2232
OH
155
171
211
OH
171 199
(-H2O, 193) 211
171.0998
293.2078
312.2256
116.9282
125.0947
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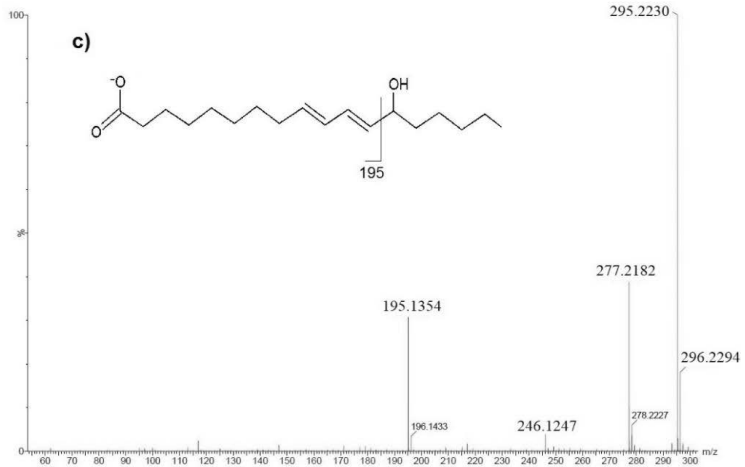
%
0
m/z


c)
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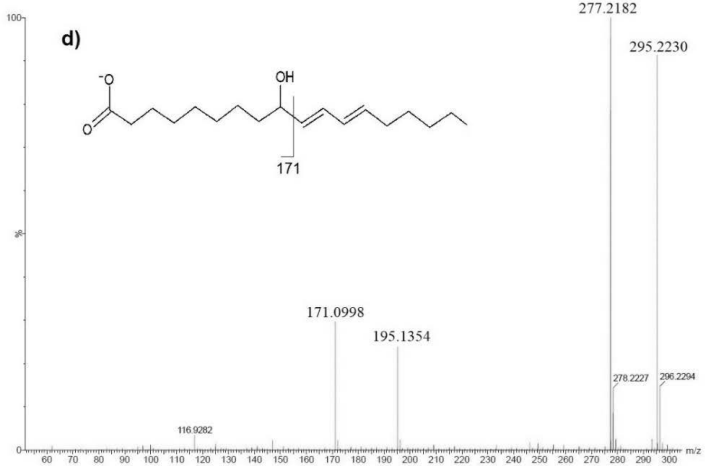
OH
195
277.2182
195.1354
296.2294
196.1433
246.1247
278.2227
100
%
0
m/z


d)
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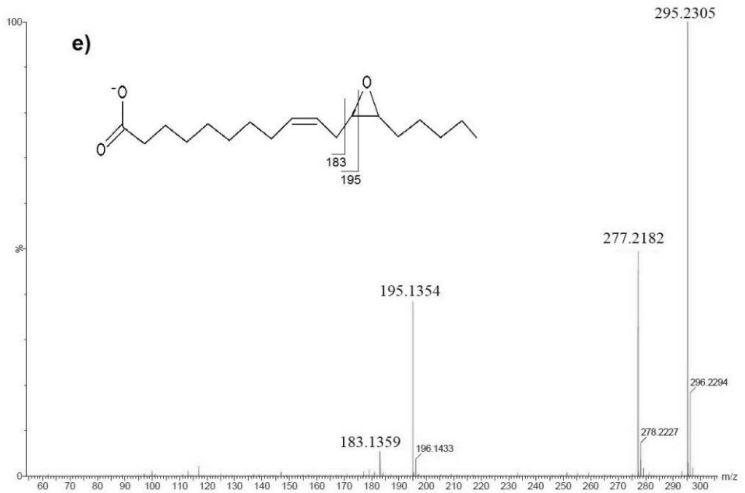
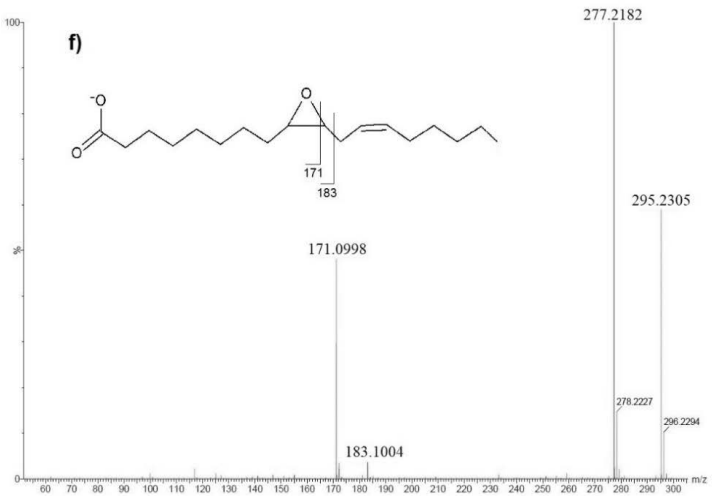
295.2230
OH
171
171.0998
195.1354
278.2227
296.2294
116.9282
100
%
0
m/z





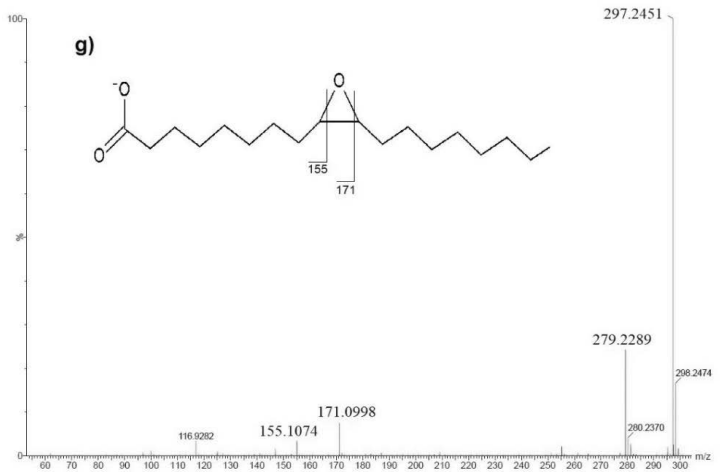


Fig. 12. Mass spectra (a–g) of the compounds C1-C7, as presented in Fig. 11. Where, a): 12,13-dihydroxy-octadec-9-enoic acid; b): a mixture of 13-hydroxy-9,10-epoxy-octadec-11-enoic acid and 9-hydroxy-12,13-epoxy-octadec-10-enoic acid; c): 13-hydroxy-octadeca-9,11-dienoic acid; d): 9-hydroxy-octadeca-10,12-dienoic acid; e): 12,13-epoxy-octadec-9-enoic acid; f): 9,10-epoxy-octadec-12-enoic acid and g): 9,10-epoxyoctadecanoic acid.

5.2 Lipid-modifying enzymes in oat and faba bean

5.2.1 Lipase activity in oat (Study I)

To measure lipase activity in oat cultivars, *p*-NPB was used as a substrate and the release of *para*-nitrophenol was measured to indicate lipase activity (μmol min^{-1} g^{-1} flour). A faba bean in-house reference sample produced a repeatable value of 6.72 ± 0.12 μmol min^{-1} g^{-1} flour (n = 13). The lipase activity ranged from 0.41–0.77 μmol min^{-1} g^{-1} flour in oat cultivar samples (Fig. 13). Significant differences were found in lipase activity among oat cultivars (Anova, $F_{3.60}$ = 125.8, $p < 0.05$). The lowest lipase activity, 0.54 ± 0.11 μmol min^{-1} g^{-1} flour on average, was found in the Alku cultivar. The highest activity, 0.71 ± 0.08 μmol min^{-1} g^{-1} flour, occurred in the Akseli cultivar. The oat lipase activity in different cultivation years also differed significantly (Anova, $F_{2.60}$ = 553.6, $p < 0.05$), with the average values being 0.48 ± 0.08, 0.64 ± 0.10 and 0.72 ± 0.05 μmol min^{-1} g^{-1} flour for the cultivation years 2012, 2013 and 2014, respectively. Thus, there was a trend toward increasing oat lipase activity between 2012 and 2014. However, in 2013, the content in the Akseli cultivar was 0.77 ± 0.02 μmol min^{-1} g^{-1} flour, which was slightly higher than that in 2014: 0.75 ± 0.02 μmol min^{-1} g^{-1} flour. Thus, all the oat cultivars possessed lipase activity, but the activity values differed in different cultivars and cultivation years.

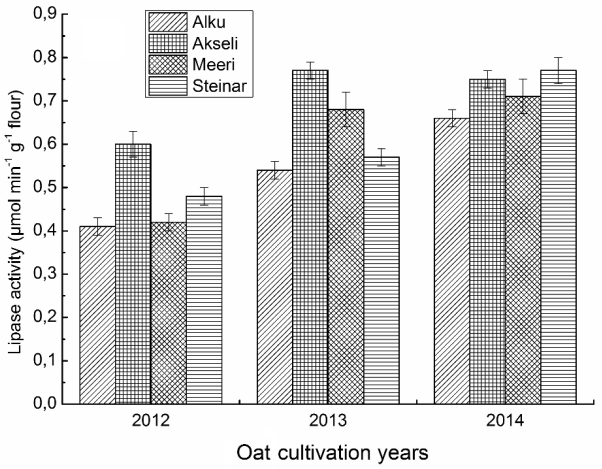


Fig. 13. Lipase activity in four oat cultivars from three cultivation years (n = 6, mean ± standard deviation).

5.2.2 Activity and substrate specificity of lipase in faba bean (Studies I & III)

5.2.2.1 Lipase activity (Study I)

The *p*-NPB substrate was also used to measure lipase activity in faba bean cultivars and the release of *para*-nitrophenol was measured to indicate lipase activity (μmol min^{-1} g^{-1} flour), and the values were determined at 4.44–7.51 μmol min^{-1} g^{-1} flour (Fig. 14). In faba bean samples, significant differences were observed among cultivars (Anova, $F_{3.60} = 259.1$, $p < 0.05$). The lipase activities in the faba bean cultivars differed more than the activities in the oat cultivars. Clearly, the Alexia cultivar had the lowest lipase activity, with a value of 4.62 ± 0.27 μmol min^{-1} g^{-1} flour on average, and Kontu had the highest activity: 7.18 ± 0.45 μmol min^{-1} g^{-1} flour. The activity values for the cultivars SSNS-1 and Fatima were 6.22 ± 0.29 and 6.85 ± 0.23 μmol min^{-1} g^{-1} flour, respectively. For the cultivation years 2010, 2011 and 2015, the average values for faba bean lipase activity were 6.16 ± 1.10, 6.28 ± 1.11 and 6.09 ± 0.94 μmol min^{-1} g^{-1} flour, respectively.

In summary, all the faba bean samples were found to possess high levels of lipase activity, and the overall values for this activity were higher than those in the oat samples. Significant differences in faba bean lipase activity were found among the various cultivars, but no statistically significant differences were found among the cultivation years (Anova, $F_{2.60} = 2.4$, $p > 0.05$).

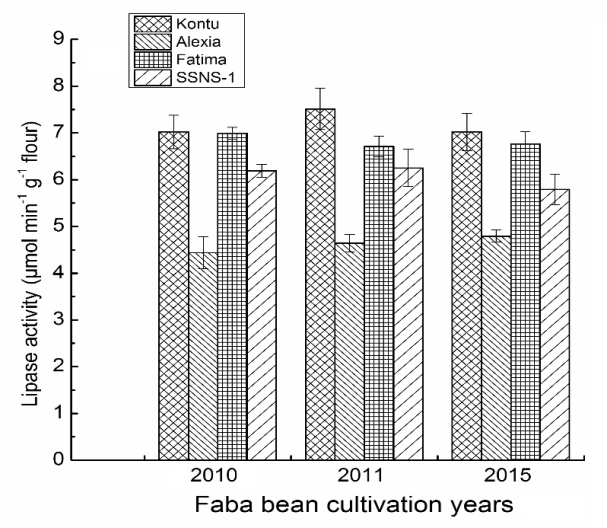


Fig. 14. Lipase activity in four faba bean cultivars from three cultivation years (n = 6, mean ± standard deviation).

5.2.2.2 Optimum pH and substrate specificity (Study III)

The optimum pH for faba bean lipase using Kontu (2016) sample was studied with *p*-NPB as a substrate at pH values of 4.5, 6, 7, 7.5 and 8. The results showed that faba bean lipase preferred alkaline conditions, with the highest activity, 4.36 ± 0.01 µmol min^{-1} g^{-1} flour, measured at pH 8 and the lower activity, *ca.* 1.5 µmol min^{-1} g^{-1} flour, measured at pHs 7.5 and 7. No activity was detected at pHs 6 and 4.5 (Fig. 15a).

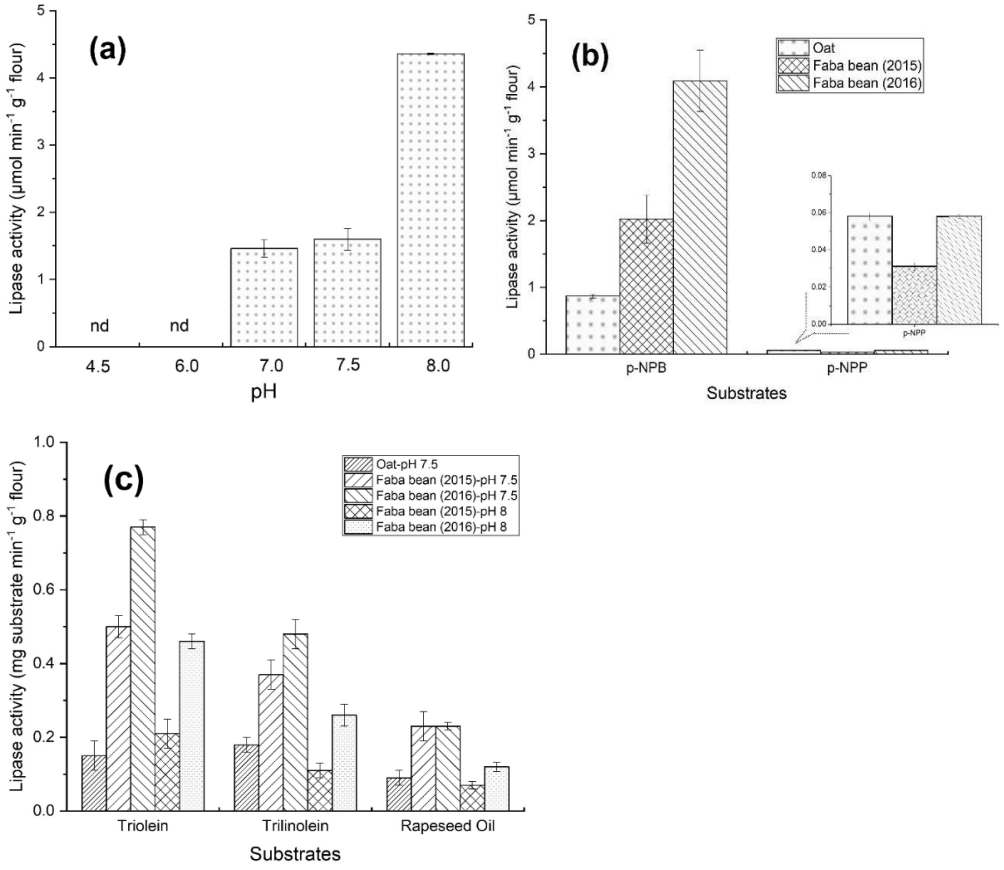


Fig. 15. Determination of optimum pH and substrate specificity of faba bean lipase. (a) optimum pH using *p*-NPB as a substrate; (b) substrate specificity using *p*-NPB and *p*-NPP at pH 8 and (c) substrate specificity using triolein, trilinolein and rapeseed oil at pHs 7.5 and 8 (n = 6, mean ± standard deviation). Oat lipase was included to compare it with faba bean lipase.

The activity of faba bean lipase was compared using two different *p*-nitrophenyl substrates, namely *p*-NPB and *p*-NPP, at pH 8.0 (Fig. 15b). In this experiment, the lipase activity of an oat sample was measured for comparison. Faba bean lipase activity was much less when *p*-NPP was used as a substrate than when *p*-NPB was used, and the activity values ranged only from

ca. 0.03–0.06 µmol min^{-1} g^{-1} flour. Similarly, the oat lipase hydrolysed more *p*-NPB substrate (0.87 ± 0.03 µmol min^{-1} g^{-1} flour) than *p*-NPP substrate (0.058 ± 0.002 µmol min^{-1} g^{-1} flour) (Fig. 15b). With the *p*-NPB substrate, the faba bean from 2016 had a higher level of lipase activity, 4.09 ± 0.46 µmol min^{-1} g^{-1} flour, than the level possessed by the faba bean from 2015, which was 2.02 ± 0.36 µmol min^{-1} g^{-1} flour (Fig. 15b). In addition, it was clear that compared with the faba bean lipase, the oat lipase hydrolysed less *p*-NPB (0.87 ± 0.03 µmol min^{-1} g^{-1} flour) and similar levels of *p*-NPP.

Faba bean lipase possessed different levels of activity toward triolein, trilinolein and RO at different pH conditions, i.e. at pHs 7.5 and 8 (Fig. 15c). Clearly, the loss of all these substrates was higher at pH 7.5 than at pH 8. When the substrates were compared, the faba bean lipase hydrolysed triolein the most, with the values being 0.77 ± 0.02 and 0.50 ± 0.03 mg triolein min^{-1} g^{-1} flour at pH 7.5 for cultivation years 2016 and 2015, respectively. The hydrolysis of RO was the least among the three substrates, with a range of only from 0.07–0.23 mg RO min^{-1} g^{-1} flour. Compared with the faba bean lipase activity, the values for lipid hydrolysis by the oat lipase were smaller, only 0.15 ± 0.04 mg min^{-1} g^{-1} flour for triolein, 0.18 ± 0.02 mg min^{-1} g^{-1} flour for trilinolein and 0.09 ± 0.02 mg min^{-1} g^{-1} flour for RO at pH 7.5.

In summary, the faba bean lipase possessed activity in neutral and alkaline pH conditions. Both the faba bean and the oat lipase catalysed hydrolysis of the *p*-NPB substrate much more efficiently than hydrolysis of the *p*-NPP substrate. Furthermore, the faba bean lipase hydrolysed more triolein and trilinolein substrates than TAGs in RO. The highest activity level occurred at pH 8 using *p*-NPB as a substrate, but more TAG substrates were hydrolysed at pH 7.5 than at pH 8. When *p*-NPB was used as a substrate, lipase activity was lower in the oat than in the faba bean. In the oat, there were only minor differences in lipase activity among the various TAG substrates, but the levels were lower than those for faba bean lipase.

5.2.3 LOX activity in oat and faba bean and properties of faba bean LOX (Studies I & III)

5.2.3.1 LOX activity in oat and faba bean (Study I)

When linoleic acid was used as a substrate for measuring LOX activity, that activity was found in faba bean cultivars (Fig. 16) but in none of the oat samples (Study I). The LOX activities among the faba bean samples ranged from 0.219 ± 0.009 to 0.330 ± 0.004 mmol min^{-1} g^{-1} flour. The Kontu and Fatima cultivars possessed the highest levels LOX activity, with average values of 0.309 ± 0.027 and 0.295 ± 0.008 mmol min^{-1} g^{-1} flour, respectively. The lowest levels of

activity 0.274 ± 0.058 mmol min^{-1} g^{-1} flour, was measured in Alexia. However, this value was only 11% lower than the highest values. The samples from cultivation years 2010 and 2015 had similar LOX activities, which were 0.299 ± 0.032 and 0.300 ± 0.029 mmol min^{-1} g^{-1} flour, respectively, while less activity was found from the year 2011, with a value of 0.278 ± 0.040 mmol min^{-1} g^{-1} flour. In summary, high levels of LOX activity were found only in the faba bean samples, and the activity differed in different cultivars (Anova, $F_{3.60} = 50.2$, $p < 0.05$) and cultivation years (Anova, $F_{2.60} = 34.2$, $p < 0.05$) and was significantly influenced by the interactions between cultivars and cultivation years (Anova, $F_{6.60} = 72.8$, $p < 0.05$).

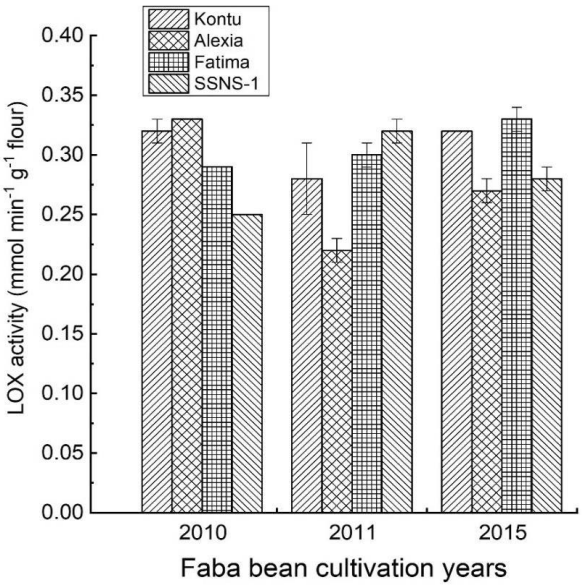


Fig. 16. LOX activity in four faba bean cultivars from three cultivation years (n = 6, mean ± standard deviation).

5.2.3.2 Characterisation of faba bean LOX (Study III)

Optimum pH

The optimum pH of faba bean LOX was investigated in Study III (Fig. 17) using linoleic acid as a substrate at pH values of 4, 5, 6, 6.8 and 8.5. The faba bean LOX possessed the highest levels of activity at pH 6, having a value of 342.8 ± 42.6 µmol g^{-1} min^{-1}. The activity values at pHs 6.8 and 5 were similar, being 183.6 ± 18.2 µmol g^{-1} min^{-1} and 162.1 ± 9.4 µmol g^{-1} min^{-1}, respectively. At pH 4, only low levels of LOX activity were found, and almost no activity was found at pH 8.5.

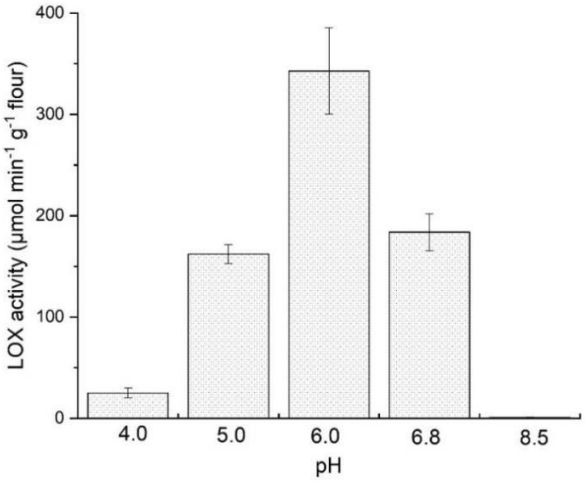


Fig. 17. Effect of pH on faba bean LOX activity using linoleic acid as a substrate (n = 6, mean ± standard deviation).

Product specificity

When the product specificity of the faba bean LOX from the cultivation years 2015 and 2016 was investigated, three hydroperoxide peaks were seen in the NP-HPLC-PDA chromatogram of LOX catalysed oxidation of linoleic acid (chromatogram not shown). The first two peaks, which had elution times of 24.8 and 26.5 min, were identified as *c,t*-13-HPOD and *t,t*-13-HPOD, respectively. The third peak, eluting at 28.7 min, was identified as a mixture of *t,c*-9-HPOD and *t,t*-9-HPOD. As shown in Table 3, in both faba bean samples, the formation of all 13-HPOD isomers together accounted for *ca.* 40% of the total hydroperoxides, while 9-HPOD accounted for *ca.* 60%.

Table 3. Distribution of hydroperoxides from faba bean LOX catalysed oxidation of linoleic acid [*].

Product	Elution time (min)	Proportion (%, year 2015)	Proportion (%, year 2016)
c,t-13-HPOD	24.8	23.3	24.3
t,t-13-HPOD	26.5	16.8	14.5
t,c-9-HPOD & *t,t*-9-HPOD	28.7	59.9	61.2

[*] Proportions given from the total peak area in two cultivation years; n = 4.

Where, *c,t*-13-HPOD = 13-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid, *t,t*-13-HPOD = 13-hydroperoxy-(9*E*,11*E*)-octadecadienoic acid, *t,c*-9-HPOD = 9-hydroperoxy-(10*E*,12*Z*)-octadecadienoic acid and *t,t*-9-HPOD = 9-hydroperoxy-(10*E*,12*E*)-octadecadienoic acid.

Effect of substrate specificity on formation of volatile products

The effect of the faba bean LOX pathway on the formation of volatile lipid oxidation product profiles was investigated by incubating faba bean enzyme extract at the LOX optimum pH 6, with various substrates: linoleic acid, linolenic acid and trilinolein (Table 4). The volatile product profiles of linoleic and linolenic acids differed and were characteristic for n-6 and n-3 PUFAs. For linoleic acid, hexanal was the major product, with a value of 94.7 ± 6.3 counts * s * 10^6, followed by 2-pentylfuran and (*E*)-2-octenal, with values of 18.8 ± 1.5 and 16.2 ± 2.1 counts * s * 10^6, respectively. For linolenic acid, 2,4-heptadienal was the major volatile product (20.6 ± 4.6 counts * s * 10^6), while other compounds, such as (*E*)-2-hexenal, (*E*,*E*,*E*)-2,4,6-nonatrienal and (*E*,*E*)-3,5-ocatadien-2-one, formed in low amounts. When using trilinolein as a substrate, only hexanal was found, in a small amount, 7.70 ± 2.56 counts * s * 10^6, showing that trilinolein was not a good substrate for faba bean LOX.

Table 4. Enzymatic formation of volatile lipid oxidation products using faba bean enzyme extract with different substrates (n = 4).

Compound	Amounts of volatile compounds (peak areas, × 10^6) with different substrates		
	Linoleic acid	**Linolenic acid**	**Trilinolein**
1-penten-3-one	nd	1.68 ± 0.26	nd
(*E*)-2-pentenal	nd	1.23 ± 0.05	nd
Hexanal	94.7 ± 6.3	2.59 ± 0.82	7.70 ± 2.56
2-heptanone	3.94 ± 0.33	nd	nd
(*E*)-2-hexenal	nd	5.38 ± 0.43	nd
Heptanal	2.36 ± 0.45	nd	nd
2-pentylfuran	18.8 ± 1.5	nd	nd
(*E*)-2-heptenal	3.41 ± 0.56	nd	nd
1-octen-3-ol	3.73 ± 0.61	nd	nd
sum of (*E*,*Z*)- and (*E*,*E*)-2,4-heptadienal	nd	20.6 ± 4.6	nd
(*E*)-2-octenal	16.2 ± 2.1	nd	nd
(*E*,*E*)-3,5-octadien-2-one	nd	4.94 ± 3.78	nd
2-nonanone	nd	1.0 ± 0.80	nd
(*E*,*E*)-2,4-nonadienal	6.28 ± 0.59	nd	nd
(*E*,*E*,*E*)-2,4,6-nonatrienal	nd	5.29 ± 2.12	nd
sum of (*E*,*Z*)- and (*E*,*E*)-2,4-decadienal	5.45 ± 0.95	nd	nd

Where, nd: not detected.

In summary, faba bean LOX had the highest activity at pH 6.0 when using linoleic acid as a substrate. In addition, the LOX produced 9-HPOD as the major hydroperoxide product. Finally, the formation of volatile compounds from linoleic acid, linolenic acid and trilinolein showed that the profiles and amounts of the volatile products from each substrate differed markedly and that more products were formed from FFA substrates than from trilinolein.

5.2.4 POX activity and substrate specificity in oat and faba bean (Study I)

When POX activity in faba bean was studied by measuring epoxide products, no activity was detected when triolein, methyl oleate or oleic acid were used as substrates. However, the oat samples possessed POX activity that catalysed the oxidation of methyl esters and FFA substrates into epoxides in the presence of cumene hydroperoxide.

Table 5. Proportions of epoxidation products [§] and substrate residues [#] of the three unsaturated fatty acid methyl esters incubated with POX from four oat cultivars in different years (n = 6).

Oat Samples	Fatty acid methyl ester substrates						
	me-oleate (%)		me-linoleate (%)			me-linolenate (%)	
Year 2012	**Epoxide[§]**	**Residue[#]**	**Epoxide 1[§]**	**Epoxide 2[§]**	**Residue[#]**	**Epoxides[§]**	**Residue[#]**
Alku	36.2 ± 0.5	59.4 ± 0.3	18.9 ± 0.1	16.3 ± 0.1	50.1 ± 0.3	25.7 ± 0.4	44.8 ± 2.0
Akseli	19.5 ± 0.3	78.2 ± 0.7	11.9 ± 0.3	10.4 ± 0.2	67.0 ± 0.7	24.1 ± 0.2	49.1 ± 0.5
Steinar	38.3 ± 0.4	56.1 ± 0.2	20.1 ± 0.0	17.1 ± 0.1	49.7 ± 0.1	25.5 ± 0.3	46.6 ± 0.5
Meeri	38.2 ± 1.2	57.6 ± 1.7	20.9 ± 0.0	17.7 ± 0.1	48.0 ± 0.4	26.5 ± 0.3	44.3 ± 0.7
Year 2013							
Alku	35.0 ± 1.7	60.6 ± 2.4	19.0 ± 0.3	15.8 ± 0.3	53.0 ± 0.8	20.6 ± 0.3	50.4 ± 1.1
Akseli	27.9 ± 1.1	69.2 ± 0.9	15.2 ± 0.2	12.7 ± 0.2	62.3 ± 0.2	22.2 ± 0.6	51.6 ± 0.5
Steinar	41.6 ± 0.4	54.0 ± 0.3	21.8 ± 0.5	18.7 ± 0.5	46.7 ± 0.4	22.6 ± 0.6	44.7 ± 1.0
Meeri	29.6 ± 0.7	67.3 ± 0.3	17.1 ± 0.2	14.8 ± 0.2	59.1 ± 1.1	23.0 ± 0.5	49.4 ± 1.0
Year 2014							
Alku	30.1 ± 0.4	67.8 ± 0.8	16.0 ± 0.3	13.3 ± 0.2	61.6 ± 0.8	23.5 ± 0.6	54.0 ± 1.4
Akseli	24.2 ± 0.3	74.2 ± 0.7	15.0 ± 0.2	12.4 ± 0.2	63.8 ± 0.7	22.1 ± 0.8	58.0 ± 0.4
Steinar	33.3 ± 0.4	60.9 ± 0.2	19.6 ± 0.2	15.9 ± 0.1	54.2 ± 0.7	23.2 ± 0.7	51.2 ± 0.8
Meeri	35.8 ± 0.4	58.3 ± 0.2	19.7 ± 0.3	16.1 ± 0.2	52.5 ± 0.2	24.4 ± 1.0	51.0 ± 1.3
Total	32.5 ± 6.2	63.6 ± 7.4	17.9 ± 2.8	15.1 ± 2.4	55.7 ± 6.6	23.6 ± 1.7	49.6 ± 4.1

[§] Proportions (%) of epoxides from methyl oleate: methyl 9,10-epoxyoctadecanoate; from methyl linoleate: 1: methyl 12,13-epoxy-octadec-9-enoate and 2: methyl 9,10-epoxy-octadec-12-enoate; and methyl linolenate: mixture of methyl mono-epoxy-octadecadienoates.

[#] Proportions (%) of substrates left from fatty acid methyl esters after incubation.

In oat, epoxide productions of 19.1–42.0% (corresponding to *ca.* 0.25–0.55 mg of epoxides when 1.3 mg of substrate was used) from fatty acid methyl esters and 34.6–70.7% (corresponding to *ca.* 0.5–1.1 mg of epoxides when 1.5 mg of substrate was used) from FFAs

were obtained after incubation with oat extract (Tables 5 and 6). Depending on the unsaturation level of the substrates, there were statistically significant differences in the amounts of epoxides measured, both among fatty acid methyl esters ($F_{2.213} = 43.3$, $p < 0.05$) and FFA substrates ($F_{2.213} = 11.9$, $p < 0.05$). The average epoxide proportions from methyl oleate, methyl linoleate and methyl linolenate were 32.5% ± 6.2%, 33.0% ± 5.2% and 23.6% ± 1.7%, respectively, and the proportions were 59.9% ± 5.4%, 58.0% ± 6.4% and 41.4% ± 3.6%, from oleic, linoleic and linolenic acids respectively. In addition, the amounts of epoxide 1 and epoxide 2 from methyl linoleate or linoleic acid were equal to each other (Tables 5 and 6). Regarding the amounts of substrate residues, the values for triunsaturated substrates ranged from 42.8–58.4% for methyl esters and from 35.6–49.7% for FFAs, whereas the values for mono- and di-unsaturated substrates ranged from 49.6–78.9% for methyl esters and from 36.7–61.8% for FFAs.

Table 6. Proportions of epoxidation products [§] and substrate residues [#] of the three unsaturated fatty acids incubated with POX from four oat cultivars in different years (n = 6).

Oat Samples	Free fatty acid substrates						
	Oleic acid (%)		Linoleic acid (%)			Linolenic acid (%)	
Year 2012	**Epoxide[§]**	**Residue[#]**	**Epoxide 1[§]**	**Epoxide 2[§]**	**Residue[#]**	**Epoxides[§]**	**Residue[#]**
Alku	67.5 ± 1.3	44.3 ± 1.1	34.1 ± 1.0	32.0 ± 1.2	37.5 ± 0.7	45.8 ± 1.4	37.4 ± 1.8
Akseli	56.8 ± 2.2	59.3 ± 2.5	26.6 ± 0.2	23.7 ± 0.7	51.7 ± 1.8	38.9 ± 0.8	48.2 ± 1.5
Steinar	63.5 ± 1.2	50.7 ± 0.8	32.9 ± 0.7	30.0 ± 1.0	43.7 ± 0.9	42.9 ± 1.2	43.2 ± 0.6
Meeri	64.0 ± 1.5	50.3 ± 0.6	30.4 ± 0.9	29.0 ± 0.7	42.5 ± 1.0	43.0 ± 0.7	42.5 ± 0.7
Year 2013							
Alku	60.4 ± 1.6	48.3 ± 1.2	31.2 ± 1.2	30.3 ± 0.4	43.5 ± 0.8	43.3 ± 0.6	37.8 ± 0.8
Akseli	51.9 ± 2.3	59.8 ± 1.1	24.4 ± 1.4	23.9 ± 0.5	53.1 ± 0.5	35.8 ± 0.9	47.4 ± 1.5
Steinar	67.4 ± 0.6	42.1 ± 1.4	35.2 ± 0.7	32.3 ± 1.5	37.7 ± 1.0	47.1 ± 1.6	37.5 ± 0.6
Meeri	56.2 ± 1.2	55.8 ± 0.9	26.7 ± 0.2	26.4 ± 0.7	50.6 ± 0.9	39.9 ± 1.8	47.1 ± 1.2
Year 2014							
Alku	58.2 ± 1.3	49.4 ± 3.1	30.5 ± 1.3	28.3 ± 0.9	44.0 ± 1.1	43.2 ± 1.7	43.3 ± 1.5
Akseli	50.7 ± 2.9	59.4 ± 2.2	24.9 ± 1.1	23.8 ± 1.1	53.8 ± 0.9	35.8 ± 1.2	48.6 ± 1.1
Steinar	60.9 ± 1.1	46.8 ± 1.0	30.3 ± 1.0	28.1 ± 0.8	43.0 ± 0.4	39.7 ± 1.1	35.8 ± 0.6
Meeri	61.1 ± 1.1	43.4 ± 1.4	31.8 ± 0.9	29.2 ± 1.0	39.0 ± 1.0	41.0 ± 1.0	36.1 ± 1.1
Total	59.9 ± 5.4	50.8 ± 6.3	29.9 ± 3.5	28.1 ± 3.1	45.0 ± 5.7	41.4 ± 3.6	42.1 ± 4.9

[§] Proportions (%) of epoxides from oleic acid: 9,10-epoxyoctadecanoic acid; from linoleic acid: 1: 12,13-epoxy-octadec-9-enoic acid and 2: 9,10-epoxy-octadec-12-enoic acid; and linolenic acid: mixture of mono-epoxy-octadecadienoic acids.

[#] Proportions (%) of substrates left from fatty acids after incubation.

Overall, significant differences in POX activities were found among oat cultivars ($F_{3.420} = 6.2$, $p < 0.05$), and the values were divided into two homogenous groups (Tukey HSD, $p < 0.05$). The first group involved the Akseli and Meeri cultivars, which had average values of 36.9% ±

11.8% and 40.8% ± 14.5%, respectively. The second group also contained the Meeri cultivar, along with the Steinar and Alku cultivars, which had average values of 44.1% ± 15.2% and 43.8% ± 14.6%, respectively. However, the cultivation year ($F_{2.420}$ = 0.98, p > 0.05) and the interactions between the cultivar and the cultivation year of the oat samples ($F_{6.420}$ = 0.72, p > 0.05) had no significant influence on oat POX activity.

In summary, high levels of POX activity were found only in oat samples, and no activity was found in faba bean. The activity in oat was primarily affected by oat cultivars. Oat POX catalysed the epoxidation of substrates at the three unsaturation levels, but the production of epoxides and the amounts of substrate residues differed remarkably. Furthermore, as substrates for oat POX, FFAs were more preferred than their fatty acid methyl esters. TAGs were not substrates, as no epoxides were found from triolein.

5.3 Epoxy and hydroxy fatty acids as non-volatile lipid oxidation products in oat (Study II)

Because oat possesses quite high levels of lipase and POX activities, Study II investigated the occurrence and formation of non-volatile lipid oxidation products in oat and their relations to the release of FFAs and volatile products as indicators of other lipid degradation reactions.

5.3.1 Occurrence of NVOFAs in flours from NHT and HT oat grains

Studying the occurrence of NVOFAs in stored NHT oat samples (F-NHT-F1, F-NHT-F2 and O-NHT-P) and in flour samples from HT oat grains (O-HT-F1 and O-HT-F2) clearly showed that NVOFAs occurred in NHT samples, but not in HT samples (Table 7). The initial total contents of NVOFAs were low in F-NHT-F1 and F-NHT-F2 samples before storage, with values of only *ca.* 280–350 μg/g flour, but the levels increased more than two-fold after six weeks of storage, and after 18 weeks, the total amounts were 2433 ± 68 and 1726 ± 43 μg/g flour for F-NHT-F1 and F-NHT-F2 samples, respectively. However, by the end of the storage period of 24 weeks, the contents of NVOFAs had decreased to 1000–1200 μg/g flour.

The major epoxy and hydroxy fatty acids (C3-C7) increased during 18 weeks of storage. However, the amounts decreased by the end of the storage period, leading to a decrease in total NVOFAs after 24 weeks (Table 7). In both samples, epoxy fatty acids were the dominant products during the initial stage of storage, but their amounts dropped sharply by the end of the storage period. There was a significant difference between the hydroxy compounds of the F-

NHT-F1 and F-NHT-F2 samples. In the F-NHT-F1 oat sample, the contents of the hydroxy compounds C3+C4 increased greatly and continuously from the initial 119 ± 3 µg/g flour to a final value of 756 ± 7 µg/g flour. In the F-NHT-F2 sample, at the beginning of six weeks of storage, the content of C3+C4 reached a peak value of 540 ± 8 µg/g flour and then dropped gradually to 223 ± 4 µg/g flour by the end of the storage period.

Table 7. Occurrence of NVOFAs in oat samples of different origins and their contents during storage at 35 °C (µg/g flour, mean ± standard deviation).

Oat samples	Sampling time	Non-volatile lipid oxidation products in oat (µg/g flour)						
		C1	C2	C3 + C4	C5	C6	C7	Total
O-HT-F1	-	nd	nd	nd	nd	nd	nd	nd
O-HT-F2	-	nd	nd	nd	nd	nd	nd	nd
O-NHT-P	-	nd	54 ± 4	565 ± 23	58 ± 2	54 ± 1	69 ± 2	800 ± 32
F-NHT-F1	0 weeks	nd	nd	119 ± 3	66 ± 2	61 ± 2	104 ± 4	350 ± 11
	6 weeks	58 ± 0.4	57 ± 0.3	667 ± 8	134 ± 5	116 ± 5	298 ± 10	1330 ± 29
	12 weeks	53 ± 2	64 ± 1	665 ± 5	139 ± 4	115 ± 4	331 ± 11	1367 ± 27
	18 weeks	51 ± 1	73 ± 2	732 ± 23	347 ± 3	349 ± 2	881 ± 37	2433 ± 68
	24 weeks	55 ± 0.2	81 ± 4	756 ± 7	61 ± 1	53 ± 1	64 ± 1	1070 ± 14
F-NHT-F2	0 weeks	nd	nd	90 ± 2	55 ± 1	55 ± 3	84 ± 1	284 ± 7
	6 weeks	73 ± 1	97 ± 5	540 ± 8	92 ± 1	79 ± 1	142 ± 3	1023 ± 19
	12 weeks	70 ± 0.4	152 ± 9	475 ± 23	183 ± 3	160 ± 6	404 ± 7	1444 ± 48
	18 weeks	95 ± 5	523 ± 16	257 ± 9	169 ± 3	177 ± 2	505 ± 8	1726 ± 43
	24 weeks	95 ± 7	495 ± 4	223 ± 4	114 ± 2	105 ± 2	196 ± 1	1228 ± 20

Where, nd: not detected; bdl: below detection limit; - : not subjected to storage experiment. The NVOFA compounds C1 and C2 were dihydroxy and epoxy-hydroxy fatty acids, C3 and C4 were monohydroxy fatty acids and C5-C7 were monoepoxy fatty acids. In detail, C1: 12,13-dihydroxy-octadec-9-enoic acid, C2: a mixture of 13-hydroxy-9,10-epoxy-octadec-11-enoic acid and 9-hydroxy-12,13-epoxy-octadec-10-enoic acid, C3: 13-hydroxy-octadeca-9,11-dienoic acid, C4: 9-hydroxy-octadeca-10,12-dienoic acid, C5: 12,13-epoxy-octadec-9-enoic acid, C6: 9,10-epoxy-octadec-12-enoic acid and C7: 9,10-epoxyoctadecanoic acid.

5.3.2 Formation of NVOFAs in flours from NHT and HT oat grains in controlled storage experiment

To study the effect of heat treatment on the formation of NVOFAs, freshly milled oat samples from NHT/HT oat grains (NHT/HT-CV1 and CV2) and freshly milled industrial scale oat samples (NHT/HT-IS) were subjected to a controlled storage experiment for four months. Only small amounts of NVOFAs were found in the freshly milled NHT-IS, NHT-CV1 and NHT-CV2. The amounts ranged from below the detection limit to 112 ± 1 µg/g flour samples. No NVOFAs were found in the flours from the HT oat grains (Tables 8 and 9). NVOFAs formed in all oat samples during storage at 40 °C, but the amounts in the flours of NHT and HT oat

grains differed greatly. In the flour from NHT oat grains, the total amount of NVOFAs increased sharply to 1700–2000 µg/g flour during a two-month storage period (Table 8), after which the amount decreased slowly but significantly. However, in all NHT oat grains, after three months of storage, the total amounts of NVOFAs had dropped off sharply, and the final values were *ca.* 690–1100 µg/g flour at the end of the storage period (Table 8). In addition, even though NVOFAs were present and increased in the HT oat samples, their formation was slow, and less than 400 µg/g flour were identified after three months of storage, at which point the values began to decrease (Table 9).

Among the various NVOFAs in the flour from NHT oat grains (C1-C7, Table 8), epoxy and hydroxy fatty acids (C3-C7) were the dominant compounds during the initial three months of storage. After that, the epoxides decreased sharply (C5-C7), but the amounts of hydroxy fatty acids (C3+C4) remained relatively stable at *ca.* 400–700 µg/g flour. Dihydroxy (C1) and epoxy-hydroxy (C2) fatty acids were formed in low amounts (*ca.* 40–120 µg/g flour) only during storage of NHT oat samples. After four months of storage, the NVOFA compounds C1-C4 became the dominant products and together accounted for the largest proportion of the total NVOFAs (Table 8). However, only epoxy and (mono-)hydroxy fatty acids (C3-C7) occurred during storage in the flours from HT oat grains (Table 9).

Table 8. Contents of NVOFAs from the flours of NHT oat grains during storage at 40 °C (µg/g flour, mean ± standard deviation).

Oat samples	Sampling time (40 °C storage)	Non-volatile lipid oxidation products in NHT oat (µg/g flour)						
		C1	C2	C3 + C4	C5	C6	C7	Total
NHT-IS	Freshly milled	nd	nd	nd	bdl	bdl	57 ± 2	57 ± 2
	1 month	nd	nd	328 ± 8	311 ± 5	303 ± 5	699 ± 7	1641 ± 25
	2 months	60 ± 2	69 ± 1	466 ± 3	243 ± 17	219 ± 13	631 ± 19	1688 ± 55
	3 months	76 ± 1	97 ± 3	564 ± 7	132 ± 17	108 ± 12	426 ± 58	1403 ± 98
	4 months	46 ± 2	67 ± 4	409 ± 27	50 ± 1	49 ± 1	71 ± 1	692 ± 36
NHT-CV1	Freshly milled	bdl	bdl	bdl	bdl	bdl	bdl	bdl
	1 month	bdl	bdl	424 ± 3	167 ± 12	141 ± 11	382 ± 0.07	1114 ± 26
	2 months	bdl	bdl	348 ± 10	352 ± 25	387 ± 14	833 ± 59	1920 ± 108
	3 months	bdl	bdl	601 ± 14	252 ± 16	187 ± 13	471 ± 16	1511 ± 59
	4 months	65 ± 1	121 ± 5	687 ± 14	68 ± 1	61 ± 0.3	107 ± 6	1109 ± 27
NHT-CV2	Freshly milled	nd	nd	49 ± 0.2	nd	nd	63 ± 0.4	112 ± 1
	1 month	47 ± 1	52 ± 0.4	441 ± 3	106 ± 3	97 ± 2	285 ± 11	1028 ± 20
	2 months	51 ± 6	63 ± 9	491 ± 17	297 ± 8	295 ± 26	789 ± 34	1986 ± 100
	3 months	80 ± 5	113 ± 8	716 ± 14	200 ± 7	159 ± 9	533 ± 29	1801 ± 72
	4 months	66 ± 2	99 ± 1	637 ± 7	55 ± 1	53 ± 0.2	76 ± 0.4	986 ± 12

Where, nd: not detected; bdl: below detection limit. The NVOFA compounds C1-C7 were the same as those listed in Table 7.

Table 9. Contents of NVOFAs from the flours of HT oat grains during storage at 40 °C (µg/g flour, mean ± standard deviation).

Oat samples	Sampling time (40 °C storage)	Non-volatile lipid oxidation products in HT oat (µg/g flour)						
		C1	C2	C3 + C4	C5	C6	C7	Total
HT-IS	Freshly milled	nd	nd	nd	nd	nd	nd	nd
	1 month	nd	nd	nd	61 ± 2	55 ± 1	62 ± 3	178 ± 6
	2 months	nd	nd	nd	53 ± 1	51 ± 1	61 ± 2	165 ± 4
	3 months	nd	nd	nd	58 ± 1	52 ± 1	61 ± 3	171 ± 5
	4 months	nd	nd	bdl	54 ± 0.3	52 ± 0.2	59 ± 2	165 ± 3
HT-CV1	Freshly milled	nd	nd	nd	nd	nd	nd	nd
	1 month	nd	nd	bdl	bdl	bdl	bdl	bdl
	2 months	nd	nd	bdl	56 ± 1	53 ± 1	70 ± 3	179 ± 5
	3 months	nd	nd	48 ± 2	67 ± 2	60 ± 1	78 ± 5	253 ± 10
	4 months	nd	nd	44 ± 1	bdl	bdl	54 ± 1	98 ± 2
HT-CV2	Freshly milled	nd	nd	nd	nd	nd	nd	nd
	1 month	nd	nd	bdl	bdl	bdl	60 ± 2	60 ± 2
	2 months	nd	nd	bdl	60 ± 1	57.6 ± 0.1	93.4 ± 0.2	210 ± 1
	3 months	nd	nd	65 ± 6	93 ± 9	76 ± 4	164 ± 18	398 ± 37
	4 months	nd	nd	44 ± 1	bdl	bdl	54 ± 0.4	98 ± 1

Where, nd: not detected; bdl: below detection limit. The NVOFA compounds C1-C7 were the same as those listed in Table 7.

5.3.3 Lipid hydrolysis and volatile lipid oxidation products in flours from NHT and HT oat grains

To relate the formation of NVOFAs and the release of FFAs and volatile compounds, changes in the amounts of FFAs and TAGs were studied during the storage of oat samples. The results showed that only small amounts of FFAs (0.8–7 mg/g flour) occurred in the freshly milled (0 month) flours from NHT oat grains and that none were found in flours from HT oat grains (Fig. 18a). However, during the first month of storage, the FFA content increased remarkably in the flours from NHT oat grains. After four months of storage, the release of FFAs had increased from their low initial values to 26 mg/g flour for NHT-CV1, 34 mg/g flour for NHT-CV2 and 32 mg/g flour for NHT-IS oat samples. In contrast, the TAGs had decreased sharply from their initial content of 30–46 mg/g to 0.7–3 mg/g flour (Fig. 18b). In the flours from HT oat grains, very low amounts of FFAs were found, and TAGs remained at high levels during storage (Fig. 18).

In the flours from NHT oat grains, the initial levels of hexanal and 2-pentylfuran were quite low, but the amounts increased sharply during two to three months of storage. The formation of 2-pentylfuran was higher than that of hexanal (Fig. 19). Compared with the volatile formation in the NHT oat samples, the increases in both hexanal and 2-pentylfuran were low in the flours from HT grains (Fig. 19). In addition, it is noteworthy that the contents of hexanal and 2-pentylfuran in NHT-IS differed markedly from the other samples and reached peak values earlier than in the other samples.

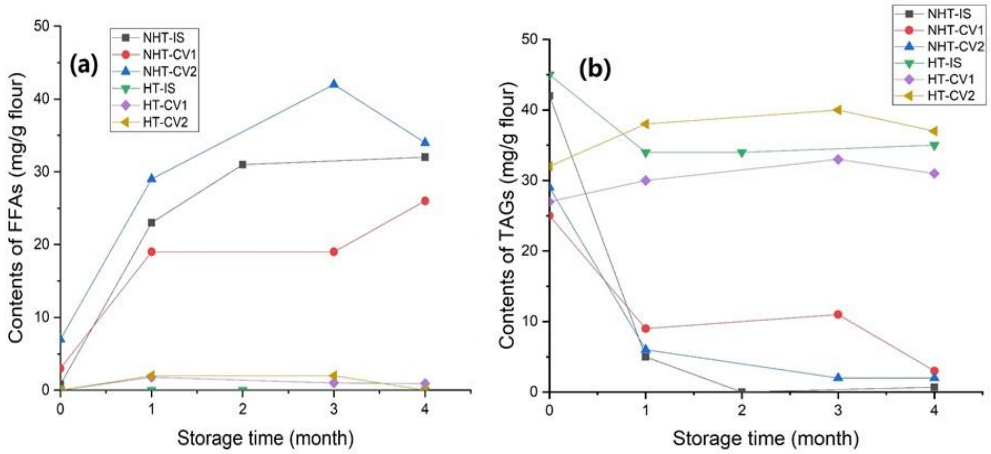


Fig. 18. Contents of a) FFAs (mg/g flour) and b) TAGs (mg/g flour) in flours from NHT and HT oat grains during storage at 40 °C.

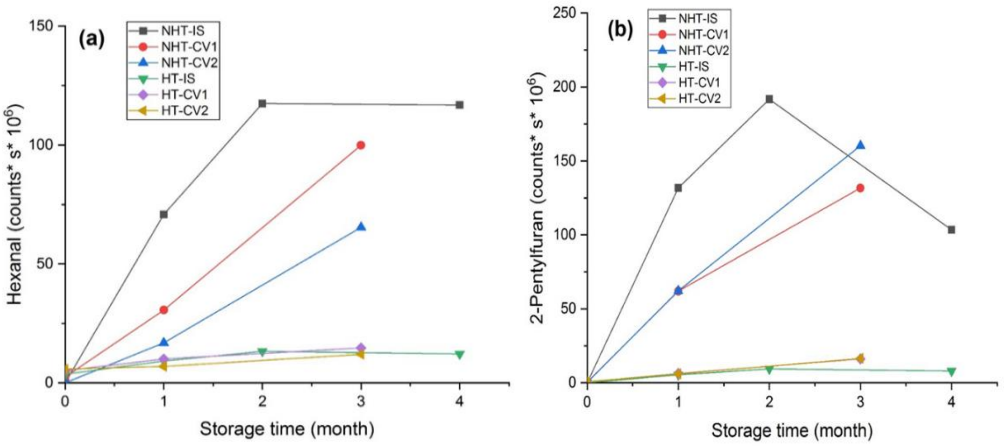


Fig. 19. Peak areas of a) hexanal and b) 2-pentylfuran in counts per second in the flours from NHT and HT oat grains during storage at 40 °C.

5.4 Effects of faba bean lipid-modifying enzymes on the formation of volatile lipid oxidation products in food models (Study III)

Rapeseed oil (RO) and rapeseed oil fatty acids (ROFA) were used to form emulsions with faba bean extract, and both the emulsions and the extract were used as food models to study the overall effects of faba bean lipid-modifying enzymes on the formation of volatile lipid oxidation products. In these food models, the amounts and types of lipids differed, and the reaction was carried out at three pH values, at which either lipase or LOX or both were expected to have activity. The measurements were made immediately after preparation of the extracts or homogenisation of the emulsions.

In the faba bean extracts (0% RO, Table 10), hexanal was the dominant lipid oxidation product in all three pH conditions of faba bean samples from both cultivation years, followed by 2-pentylfuran, 1-hexanol and 1-octen-3-ol. However, the amounts of the major products at different pHs differed markedly. At pH 6.4, the production of hexanal ranged from 6.9–7.4 counts * s * 10^6 for both faba bean samples, while at pH 5, the values increased to 10.7–13 counts * s * 10^6, and when incubated at pH 8, they were quite low, only *ca.* 2.5 counts * s * 10^6.

However, when RO was included in the system to make an emulsion with faba bean extract (3% RO and 5% RO), the amounts of volatile products increased remarkably in emulsions prepared from both faba bean samples (Table 10). The 2,4-heptadienal and 2-pentenal were found only in emulsions, not in extracts. The increase of 2-pentylfuran and 1-octen-3-ol was quite small,

even less than in the faba bean extract. At pH 6.4, the addition of 3% RO increased the amounts of volatile products by two-fold and the addition of 5% RO increased them by three-fold compared to their respective extracts. However, at pHs 5 and 8, the addition of RO did not greatly increase the amounts of volatile products (Table 10).

When ROFA emulsions (3% ROFA and 5% ROFA) were used as a food model, the formation of volatile products was greater than in the RO emulsions using both faba bean samples (Table 11). Compared with the amounts of volatile compounds formed in the RO emulsions, the production in the ROFA emulsions was 4-to-8–fold at pH 5, 2-to-3–fold at pH 6.4 and 6-to-15–fold at pH 8. When the formation of volatiles at various reaction pHs was compared, the volatile products were found to have formed most abundantly at pH 6.4, followed by pH 5, and the amounts formed were quite low at pH 8 (Table 11). At all pHs, hexanal was the major volatile product in the emulsions. At pH 6.4, its amount in the 3% ROFA emulsion was *ca.* 28–33 counts * s * 10^{6}, which was even higher than in the 5% ROFA emulsion. However, at pH 5, the content of hexanal with the addition of ROFA was slightly lower than at pH 6.4, and the lowest amounts were produced at pH 8 (Table 11).

Table 10. Contents of volatile lipid oxidation products in faba bean extract (0% RO) and rapeseed oil emulsions (3% RO and 5% RO) containing faba bean extract at selected pHs (3 replicate measurements; nd: not detected).

Compound	Faba Bean (Kontu 2016)								
	pH 5 (counts * s * 10^6)			pH 6.4 (counts * s * 10^6)			pH 8 (counts * s * 10^6)		
	0%RO	3%RO	5%RO	0%RO	3%RO	5%RO	0%RO	3%RO	5%RO
1-penten-3-one	nd	1.4 ± 0.3	2.9 ± 0.6	nd	0.2 ± 0.09	0.3 ± 0.07	nd	nd	nd
(*E*)-2-pentenal	nd	0.9 ± 0.2	2.4 ± 0.4	nd	4.6 ± 0.6	6.3 ± 0.6	nd	0.4 ± 0.1	0.2 ± 0.0
Hexanal	13.0 ± 0.2	12.2 ± 2.6	12.4 ± 0.8	7.4 ± 0.9	17.2 ± 1.4	21.5 ± 1.6	2.4 ± 0.5	3.5 ± 0.2	2.2 ± 0.3
(*E*)-2-hexenal	0.8 ± 0.2	0.6 ± 0.1	1.0 ± 0.1	nd	0.6 ± 0.1	0.6 ± 0.1	nd	nd	nd
1-hexanol	0.6 ± 0.1	nd	0.3 ± 0.1	4.5 ± 1	7.1 ± 1.2	7.6 ± 2.2	0.1 ± 0.0	0.2 ± 0.0	nd
Heptanal	nd	0.5 ± 0.2	0.5 ± 0.1	nd	2.5 ± 0.3	4.7 ± 1.2	nd	nd	nd
2-pentylfuran	1.4 ± 0.1	nd	0.1 ± 0.0	4.2 ± 1.1	1.6 ± 0.1	2.3 ± 0.2	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
(*E*)-2-heptenal	0.2 ± 0.1	0.7 ± 0.1	0.9 ± 0.1	nd	2.7 ± 0.2	4.5 ± 0.2	nd	nd	nd
1-octen-3-ol	1.6 ± 0.7	0.2 ± 0.03	0.2 ± 0.0	3.1 ± 1.4	3.2 ± 0.6	3.4 ± 0.4	0.5 ± 0.1	0.6 ± 0.1	0.3 ± 0.1
Octanal	nd	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.8 ± 0.1	nd	nd	nd
sum of (*E*,*Z*)- and (*E*,*E*)-2,4-heptadienal	nd	nd	0.3 ± 0.0	nd	1.4 ± 0.4	2.8 ± 0.3	nd	nd	nd
(*E*)-2-Octenal	nd	0.2 ± 0.0	0.3 ± 0.0	nd	1.6 ± 0.3	2.0 ± 0.1	nd	nd	nd
Nonanal	0.2 ± 0.1	0.8 ± 0.0	0.7 ± 0.1	2.0 ± 0.8	3.9 ± 0.5	4.1 ± 0.4	0.3 ± 0.1	1.0 ± 0.0	0.4 ± 0.1
(*E*,*E*)-3,5-octadien-2-one	nd	nd	nd	nd	nd	nd	nd	nd	nd
Octanoic acid	nd	nd	nd	1.7 ± 0.5	0.8 ± 0.2	1.4 ± 0.3	nd	nd	nd
Compound	**Faba Bean (Kontu 2015)**								
	pH 5 (counts * s * 10^6)			**pH 6.4 (counts * s * 10^6)**			**pH 8 (counts * s * 10^6)**		
	0%RO	3%RO	5%RO	0%RO	3%RO	5%RO	0%RO	3%RO	5%RO
1-penten-3-one	nd	2.1 ± 0.7	4.1 ± 1.8	nd	1.3 ± 0.3	1.4 ± 0.7	nd	1.1 ± 0.0	1.0 ± 0.4
(*E*)-2-pentenal	nd	2.5 ± 0.8	3.8 ± 1.0	nd	3.5 ± 0.6	6.1 ± 0.7	nd	0.2 ± 0.0	0.2 ± 0.0
Hexanal	10.7 ± 0.4	14.4 ± 1.2	10.9 ± 3.7	6.9 ± 0.8	15.5 ± 3.5	22.2 ± 4.5	2.5 ± 0.5	3.1 ± 0.2	2.4 ± 0.1
(*E*)-2-hexenal	0.4 ± 0.1	1.0 ± 0.3	0.8 ± 0.2	nd	1.1 ± 0.1	2.3 ± 0.4	nd	nd	nd
1-hexanol	nd	0.4 ± 0.2	0.1 ± 0.0	5.6 ± 0.4	6.9 ± 2.4	7.6 ± 2.0	nd	0.3 ± 0.1	0.1 ± 0.0
Heptanal	nd	1.0 ± 0.4	0.9 ± 0.3	nd	2.2 ± 0.8	3.8 ± 0.9	nd	nd	nd
2-pentylfuran	0.5 ± 0.1	0.1 ± 0.06	0.1 ± 0.0	3.0 ± 0.2	0.6 ± 0.2	0.7 ± 0.2	0.1 ± 0.0	0.1 ± 0.0	0.03 ± 0.01
(*E*)-2-heptenal	nd	1.7 ± 0.5	1.2 ± 0.2	nd	1.7 ± 0.5	3.0 ± 0.5	nd	nd	nd
1-octen-3-ol	2.0 ± 0.6	0.7 ± 0.1	0.8 ± 0.1	2.3 ± 0.0	2.3 ± 0.8	2.8 ± 0.6	0.4 ± 0.1	0.5 ± 0.1	0.2 ± 0.06
Octanal	0.04 ± 0.01	0.9 ± 0.3	0.5 ± 0.1	nd	0.4 ± 0.1	0.7 ± 0.1	nd	nd	nd
sum of (*E*,*Z*)- and (*E*,*E*)-2,4-heptadienal	nd	0.7 ± 0.2	0.5 ± 0.1	nd	0.8 ± 0.3	2.2 ± 0.4	nd	nd	nd
(*E*)-2-Octenal	nd	0.7 ± 0.3	0.3 ± 0.1	nd	1.1 ± 0.2	1.6 ± 0.2	nd	nd	nd
Nonanal	0.3 ± 0.1	1.7 ± 0.5	1.0 ± 0.4	nd	2.9 ± 0.6	3.4 ± 0.3	0.3 ± 0.1	0.5 ± 0.1	0.2 ± 0.05
(*E*,*E*)-3,5-octadien-2-one	nd	nd	nd	nd	nd	nd	nd	nd	nd
Octanoic acid	nd	nd	nd	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	nd	nd	nd

Table 11. Contents of volatile lipid oxidation products in rapeseed oil fatty acid emulsions (3% ROFA and 5% ROFA) containing faba bean extract in selected pHs (3 replicate measurements; nd: not detected).

Compound	Faba Bean (Kontu 2016)					
	pH 5 (counts * s * 10^6)		pH 6.4 (counts * s * 10^6)		pH 8 (counts * s * 10^6)	
	3%ROFA	5%ROFA	3%ROFA	5%ROFA	3%ROFA	5%ROFA
1-penten-3-one	5.5 ± 0.6	10.4 ± 2.2	5.4 ± 0.1	7.2 ± 1.1	0.5 ± 0.1	1.5 ± 0.5
(*E*)-2-pentenal	4.7 ± 0.7	7.9 ± 0.7	7.5 ± 0.7	10.1 ± 0.8	4.4 ± 1.3	7.3 ± 1.8
Hexanal	19.4 ± 2.8	20.2 ± 3.1	33.0 ± 3.9	29.0 ± 2.7	11.7 ± 2.8	12.0 ± 3.5
(*E*)-2-hexenal	2.4 ± 0.6	3.2 ± 0.1	13.0 ± 3.7	11.2 ± 1.1	4.5 ± 0.8	3.9 ± 0.7
1-hexanol	nd	nd	4.3 ± 1.8	1.6 ± 0.3	0.2 ± 0.1	0.2 ± 0.0
Heptanal	2.4 ± 0.3	3.3 ± 0.4	3.0 ± 1.0	4.1 ± 0.6	1.6 ± 0.9	2.7 ± 0.8
2-pentylfuran	0.8 ± 0.3	0.7 ± 0.1	7.9 ± 0.3	5.4 ± 0.5	4.2 ± 1.1	4.5 ± 0.6
(*E*)-2-heptenal	7.9 ± 1.4	9.5 ± 0.2	11.1 ± 3.5	12.9 ± 1.0	1.1 ± 0.4	2.4 ± 0.5
1-octen-3-ol	1.8 ± 0.2	2.5 ± 0.3	10.1 ± 4.9	9.3 ± 0.8	2.3 ± 0.6	2.8 ± 0.4
Octanal	1.5 ± 0.2	1.7 ± 0.1	4.1 ± 1.9	2.5 ± 0.2	nd	nd
sum of (*E*,*Z*)- and (*E*,*E*)-2,4-heptadienal	13.0 ± 2.8	15.0 ± 0.8	8.0 ± 2.3	15.6 ± 1.3	0.8 ± 0.2	3.2 ± 0.5
(*E*)-2-Octenal	4.5 ± 1.2	5.2 ± 0.5	1.6 ± 0.3	2.0 ± 0.1	1.0 ± 0.5	1.9 ± 0.5
Nonanal	3.6 ± 0.5	3.1 ± 0.2	10.0 ± 2.6	6.4 ± 0.3	2.8 ± 0.7	2.7 ± 0.3
(*E*,*E*)-3,5-octadien-2-one	2.0 ± 0.7	1.7 ± 0.3	2.4 ± 0.1	2.5 ± 0.2	0.4 ± 0.2	1.0 ± 0.3
Octanoic acid	0.8 ± 0.4	0.7 ± 0.1	2.4 ± 1.0	2.3 ± 0.1	0.7 ± 0.0	0.9 ± 0.1
Compound	**Faba Bean (Kontu 2015)**					
	pH 5 (counts * s * 10^6)		pH 6.4 (counts * s * 10^6)		pH 8 (counts * s * 10^6)	
	3%ROFA	5%ROFA	3%ROFA	5%ROFA	3%ROFA	5%ROFA
1-penten-3-one	7.7 ± 1.0	13.3 ± 3.2	5.2 ± 0.4	8.5 ± 1.2	0.7 ± 0.3	0.4 ± 0.2
(*E*)-2-pentenal	5.7 ± 1.0	12.6 ± 1.8	6.4 ± 0.6	9.3 ± 1.7	4.6 ± 1.2	5.1 ± 0.3
Hexanal	22.4 ± 0.1	20.0 ± 2.7	28.1 ± 4.3	26.4 ± 2.3	7.0 ± 1.8	7.2 ± 1.3
(*E*)-2-hexenal	4.0 ± 1.0	7.3 ± 1.2	8.1 ± 0.4	8.3 ± 0.5	5.4 ± 1.5	2.9 ± 0.7
1-hexanol	nd	nd	2.0 ± 0.2	1.8 ± 0.0	1.1 ± 0.3	0.3 ± 0.1
Heptanal	4.3 ± 1.1	8.4 ± 2.3	3.1 ± 0.4	3.0 ± 0.7	1.8 ± 0.7	3.0 ± 1.2
2-pentylfuran	1.0 ± 0.2	1.6 ± 0.3	5.4 ± 0.6	3.8 ± 1.2	4.3 ± 1.3	4.4 ± 0.9
(*E*)-2-heptenal	9.0 ± 1.5	9.5 ± 0.2	8.0 ± 1.8	9.6 ± 2.2	1.0 ± 0.3	2.5 ± 0.4
1-octen-3-ol	2.4 ± 0.6	3.3 ± 0.7	5.5 ± 0.4	6.2 ± 1.7	2.3 ± 0.6	3.5 ± 0.7
Octanal	2.0 ± 0.3	2.6 ± 0.4	3.3 ± 0.5	2.5 ± 0.6	nd	nd
sum of (*E*,*Z*)- and (*E*,*E*)-2,4-heptadienal	6.4 ± 1.3	10.2 ± 2.4	14.4 ± 2.1	25.0 ± 2.2	1.7 ± 0.4	1.8 ± 0.4
(*E*)-2-Octenal	4.7 ± 1.3	8.7 ± 2.2	6.0 ± 1.5	6.7 ± 1.4	1.2 ± 0.5	2.2 ± 0.5
Nonanal	3.9 ± 0.5	3.9 ± 0.6	7.5 ± 0.8	5.6 ± 1.1	3.7 ± 0.8	3.0 ± 0.4
(*E*,*E*)-3,5-octadien-2-one	1.1 ± 0.3	1.7 ± 0.4	1.1 ± 0.5	1.8 ± 0.4	0.5 ± 0.1	1.4 ± 0.3
Octanoic acid	0.8 ± 0.1	1.1 ± 0.0	1.3 ± 0.1	1.5 ± 0.2	0.4 ± 0.1	0.8 ± 0.1

6 DISCUSSION

6.1 Evaluating methods of measuring POX activity and analysing NVOFAs

6.1.1 GC and UHPLC methods of measuring POX activity (Study I)

POX activity was measured by detecting the epoxidation products from substrates using cumene hydroperoxide as an oxidant. The incubation was conducted with enzyme extract at pH 7 for 1 h at room temperature. Only monoepoxides were found as oxidation products. According to Meesapyodsuk and Qiu (2011), the optimum pH for oat POX activity was pH 7 using oleic acid as a substrate and cumene hydroperoxide as an oxygen donor, based on which the method used in the present study was further developed. A short reaction time (1 h) for incubation of substrates with POX was preferred, because the monoepoxides formed had the potential to convert further into other compounds (e.g. diepoxy fatty acids) during long incubation times (e.g. 24 hours) (Piazza et al. 2003b). A GC-FID/MS method was used to analyse epoxy products from fatty acid methyl esters, including methyl oleate, methyl linoleate and methyl linolenate, and triolein. In addition, to avoid a lengthy derivatisation process prior to GC, a UHPLC-ELSD/MS method was developed to directly analyse epoxy products from FFA substrates, oleic, linoleic and linolenic acids.

6.1.1.1 GC-FID/MS method

The GC-FID/MS method separated the epoxy fatty acid methyl esters produced by POX from substrates, and the epoxide products were further identified using GC-MS (Fig. 9, Table 2). All the formed products were mono-epoxy fatty acid methyl esters, which were eluted after their methyl ester substrates. One study used a GC method to examine oat POX activity and substrate specificity by measuring epoxides from various FFA substrates after methylation and also investigate the optimum pH of POX using methyl oleate as a substrate (Meesapyodsuk and Qiu 2011). Another study observed the accumulation of oxygenated fatty acids using a method based on GC (Doehlert et al. 2010), but the focus of that study was on the overall epoxidation products oxidised from lipids during oat storage. Thus, the GC-FID/MS method used in the present study provided a new approach to measuring POX activity by detecting proportions of epoxides from the initial fatty acid methyl esters.

6.1.1.2 UHPLC-ELSD/MS method

The UHPLC-ELSD/MS method separated, quantified and identified the epoxy fatty acid products formed from FFA substrates (Fig. 10, Table 2). Using this method, only mono-epoxy fatty acids were detected, and the unsaturated epoxides eluted earlier than the saturated ones.

The elution order and fragmentation pathways were in line with those presented in another study that used HPLC-ELSD/MS (Orellana-Coca et al. 2005). In the present study, the epoxide products from oleic, linoleic and linolenic acids were determined using an RP-HPLC-ELSD method, and the structures of the epoxides were determined using MS. Other studies used LC methods to measure the epoxidation products formed from FFAs by POX (Piazza et al. 2003b; Orellana-Coca et al. 2005), and one of them tested the formation of epoxides using immobilised oat POX and a variety of substrates, including oleic, linoleic and linolenic acids (Piazza et al. 2003b). However, that study focused mainly on the product specificity of oat POX, and the epoxidation products from FFA substrates were methylated before LC analysis (Piazza et al. 2003b). The method used in the present research successfully measured the products from various substrates and proved to be a reliable approach to measuring POX activity using the proportions of epoxides formed from initial FFA substrates.

In summary, the repeatable results regarding the formation of methyl 9,10-epoxyoctadecanoate from methyl oleate, determined using the GC-FID method, and the formation of 9,10-epoxyoctadecanoic acid from oleic acid, determined using the UHPLC-ELSD method, when the in-house reference samples were analysed, confirmed that the analytical levels of the methods developed were stable and reliable. Thus, the GC and UHPLC methods reliably separated and quantified epoxy fatty acid products produced by oat POX. The amount of epoxide(s) from each substrate was collected, and the POX activity was expressed as the proportions of epoxides formed from the initial substrates. In addition to measuring POX activity, these methods were used to investigate the substrate specificity of POX by measuring the amount of products formed from various substrates. Lastly, the methods provided a reliable way to further study POX activity in other food materials.

6.1.2 UHPLC-ELSD/MS method for analysing NOVFAs in oat (Study II)

To study the occurrence and formation of NVOFAs, a method that included ASE, SPE and UHPLC-ELSD/MS was developed to extract, purify and analyse the oxidised fatty acids in oat samples. The ASE method was shown to provide high extraction yields of lipids from oat. The SPE method was shown to remove the TAGs and most FFAs from the lipid extract. The good recovery values of the epoxy and hydroxy fatty acids added to the oat flour verified that the solvents efficiently extracted NVOFAs and that the oxidised fatty acids remained stable during extraction and SPE fractionation. Furthermore, after the extraction and purification process, the

UHPLC-ELSD/MS method developed quantified and identified the NVOFAs, epoxy and hydroxy fatty acids.

All the NVOFAs collected after SPE fractionation were separated within the elution time of 28 min using UHPLC-ELSD, except for the compounds marked as peaks C3 and C4 at the elution time 9.2 min and two epoxy-hydroxy compounds co-eluted in peak C2 (Fig. 11). The fragmentation of 12,13-dihydroxy-octadec-9-enoic acid (C1, Fig. 11) was identified in line with studies by Newman et al. (2002) and Levandi et al. (2009). This compound was suggested as being hydrolysed by epoxide hydrolase from 12,13-epoxy-octadec-9-enoic acid (Hamberg and Hamberg 1996). C2 (Fig. 11) was identified as a mixture of two epoxy-hydroxy fatty acid isomers, and the fragments formed were similar to those studied by Huang and Schwab (2012) and Yuan et al. (2018). The formation of these epoxy-hydroxy fatty acids in oat samples has been suggested as being based on POX-catalysed intramolecular epoxidation of hydroperoxides containing two double bonds, as proposed in Fig. 5 and as reported by Piazza et al. (1999) and Hanano et al. (2006). Two conjugated mono-hydroxy compounds, 13-hydroxy-octadeca-9,11-dienoic acid and 9-hydroxy-octadeca-10,12-dienoic acid (C3 and C4, Fig. 11) were co-eluted, which was also observed by Schneider et al. (1997) using an RP-HPLC method. The C3 and C4 compounds were POX-catalysed lipid oxidation products of the hydroperoxides from linoleic acid (Hamberg and Hamberg 1996). Mono-epoxy fatty acids, 12,13-epoxy-octadec-9-enoic acid and 9,10-epoxy-octadec-12-enoic acid (C5 and C6, Fig. 11) from linoleic acid and 9,10-epoxysteric acid (C7) from oleic acid were found in native oat flours, and all were typical epoxides oxidised by oat POX (Hamberg and Hamberg 1996). Most of the NVOFAs were oxygenated from oleic and linoleic acids, the most abundant UFAs in oat.

Previously, only a few studies have focused on extracting and analysing NVOFAs from food matrices (Leonova et al. 2008; Doehlert et al. 2010; Yao and Schaich 2014). GC methods have been used to analyse the oxidised fatty acid products in foods (Leonova et al. 2008; Doehlert et al. 2010). Leonova et al. (2008) analyzed the fatty acid composition from various oat cultivars, and some epoxy and hydroxy fatty acids, such as 7-hydroxyhexadecanoic acid and epoxides from oleic and linoleic acids, by GC after methylation. However, a GC method usually requires lengthy derivatisation steps that may degrade the products. An LC-MS/MS method was used to study the lipid oxidation products from wheat varieties directly after lipid extraction without purification (Levandi et al. 2009). However, it is not recommended to analyse lipid extracts without removing neutral lipids using RP-LC, because neutral lipids may interfere with the

analysis by adsorbing to the column. Therefore, the present research provided an alternative approach to extract, purify and analyse the oxidised lipid products by LC from foods.

In summary, the developed method including ASE extraction, SPE fractionation and UHPLC-ELSD/MS analysis enabled identification of several NVOFAs, gave high recoveries of added NVOFAs and repeatable values from the oat in-house reference sample. Overall, the method developed was able to quantitatively analyse the NVOFAs formed in native oat matrix and also helped to increase understanding of the formation and degradation reaction routes of POX-catalysed lipid oxidation products in oat.

6.2 Evaluating lipid-modifying enzymes in oat and faba bean

6.2.1 Lipase activity was found in both oat and faba bean

Both oat and faba bean cultivars possessed significant lipase activity (Figs. 13 and 14). Using *p*-NPB as a substrate, the activity was shown to vary among oat cultivars (Fig. 13). In contrast, an increasing trend was observed in oat lipase activity from 2012 to 2014, which may indicate that lipase activity can be negatively influenced by long-term storage. In addition, growing and harvesting conditions could also affect the activity of oat lipase. When *p*-NPB was used as a substrate, the faba bean cultivars had much higher lipase activity than the oat cultivars (Fig. 14). Furthermore, there was more variation in the results for the lipase activity of faba bean in the samples analysed, and the large variation within cultivation years suggested that this variation was due mainly to differences among cultivars. The repeatable results for the faba bean in-house reference samples, which were included in each batch of analysis, confirmed that the analytical level was stable and that the variation in the results was not caused by variations in measuring the lipase activity. Studies have well characterised the effect of lipase in oat (Ekstrand et al. 1992; Piazza et al. 1992; Lehtinen et al. 2003; Decker et al. 2014), but knowledge is limited regarding the lipase activity in faba bean. One old study investigated lipase activity from small faba beans using various *p*-nitrophenyl fatty acyl esters (Henderson et al. 1981), and another examined the activity and characterisation of lipase in faba bean (Dundas et al. 1978). The results of the present research showed that high lipase activity should be properly controlled during processing of oat and faba bean. Because in combination with some other lipid-modifying enzyme activities, e.g. LOX activity, there is a high risk of undesirable flavours in oat and faba bean foods.

Because measuring lipase activity using *p*-NPB as a substrate has been criticised as being nonspecific (Gilham and Lehner 2005), a long-chain fatty acid ester (*p*-NPP), triolein, trilinolein and RO TAGs were used as substrates when investigating the substrate specificity of faba bean lipase (Study III). The significantly higher hydrolysis of *p*-NPB than of *p*-NPP at pH 8 (Fig. 15b) showed that faba bean lipase preferred the short-chain fatty acid ester more than the long-chain one. The results of optimum pH measurement using *p*-NPB showed that the lipase possessed higher activity at pH 8.0 than at lower pH values, and no activity was observed below pH 6 (Fig. 15a). Older studies found that faba bean lipase had the highest activity at an alkaline pH, 8.5, using tributyrin or olive oil (Dundas et al. 1978) and *p*-NPP or *p*-nitrophenyl laurate (Henderson et al. 1981) as substrates. In the present research, faba bean lipase also catalysed the hydrolysis of triolein, trilinolein and RO in alkaline conditions, but the reaction rates were greatly affected by the TAG substrate and reaction pH (Fig. 15c). Lipase in faba bean from both cultivation years had higher activity than in oat (Fig. 15c). At both pHs 7.5 and 8, faba bean lipase seemed to catalyse hydrolysis of homogenous TAGs, triolein and trilinolein better than hydrolysis of RO TAGs, indicating that the structure of TAGs may influence enzyme reactions. An older study reported that faba bean lipase was more active toward the glycerides of short-chain fatty acids than toward those of long-chain fatty acids (Dundas et al. 1978). However, that study compared the hydrolysis of tributyrin only to olive or corn oils. Thus, more studies are needed on the substrate specificity of faba bean lipase. Furthermore, when comparing the activity at different pHs, more TAGs were hydrolysed by faba bean lipase at pH 7.5 than at pH 8 (Fig. 15c), which was opposite to the finding that the maximum activity was obtained at pH 8 using *p*-NPB as a substrate (Fig. 15a). The reason for this may be that *p*-NPB serves as a non-specific substrate for several hydrolysing enzymes.

The high optimum pH for faba bean lipase showed that there might be a risk for lipid degradation, especially when using faba bean as an ingredient in mildly alkaline conditions. However, these conditions are not commonly found in food systems. On the other hand, the hydrolysis may begin in faba bean ingredients during storage. The water activity in foods is an important factor that affects lipase activity, and the kinetics of lipase reactions depend on the moisture content. In some oat products, the lipid-hydrolysis process was found to be slow in the flours from NHT and HT oat grains at relatively low humidity but dramatically higher in both flours when they had been pre-treated with water (a 1-g flour sample soaked with 5 ml of water) (Lehtinen et al. 2003). In wheat bran, lipase possessed the highest activity at moisture contents of about 20%, but the activity was relatively low when the content was either extremely

low (less than 10%) or higher than 30% (Rose and Pike 2006). However, studies are still lacking concerning the effects of water activity on faba bean lipase activity in dry food matrices. The results of Study III showed that when faba bean extract was mixed with TAGs in RO to form an emulsion at pH 6.4, the lipase possessed some activity. An older study found that faba bean lipase hydrolysed olive oil and corn oil in emulsions at pH 8.5 (Dundas et al. 1978).

In summary, both oat and faba bean possessed lipase activity, and that activity varied among cultivars and cultivation years. Both oat and faba bean lipase hydrolysed more of the short-chain fatty acid ester (*p*-NPB) than of the long-chain one (*p*-NPP), which might be due to the non-specific hydrolysis of *p*-NPB. Faba bean lipase hydrolysed more triolein than trilinolein or RO TAGs, and its activity toward all these substrates was higher than that of oat lipase. By studying various substrates, the present research confirmed that true lipase activity was present in faba bean and catalysed the hydrolysis of TAGs. Finally, controlling lipase activity is necessary when both oat and faba bean are used as food ingredients, because high lipase activity may begin lipid hydrolysis as long as the reaction conditions in foods are suitable.

6.2.2 LOX activity was detected only in faba bean

Study I found no LOX activity in any of the oat cultivar samples when using linoleic acid as a substrate. However, oat may possess low LOX activity (Lehtinen and Kaukovirta-Norja 2011), which may cause lipid-derived problems by producing hydroperoxides, which can further affect the quality of oat products. Thus, oat LOX activity should not be ignored during oat processing. In this thesis, high LOX activity was found in faba bean cultivars (Fig. 16), which was also observed in the faba bean cultivar samples in other studies (Alobaidy and Siddiqi 1981; Chang and McCurdy 1985). According to these studies, faba bean possessed medium level of LOX activity compared with some other legumes, such as soybean and lentil. The faba bean LOX activity found in Study I was comparable to the level (0.44 mmol min^{-1} g^{-1} flour) previously found in faba bean in Finland (Jiang et al. 2016). In addition, the significantly different LOX activity values found in the faba bean samples indicated that the cultivars and growing conditions greatly affected faba bean LOX activity.

At pH 6, LOX possessed its highest activity, which was two-fold compared with the activity at pHs 5 and 6.8 (Fig. 17). These results were in line with those of Clemente et al. (2000), who reported that the optimum pH for LOX isolated from faba bean was about 5.8. Although the lipid content in faba bean is quite low, lipids should also be considered, because even low

concentrations of secondary lipid oxidation products may produce distinct disadvantages (Akkad et al. 2019), and when oil is added, as in emulsions, the risk of enzymatic lipid degradation increases. To identify the structure of the hydroperoxides formed from linoleic acid by faba bean LOX, soybean LOX-1 was used to produce these hydroperoxides, whose identities and elution orders were already known (Perraud and Kermasha 2000). Study III found that 9-HPOD was the major hydroperoxide formed by faba bean LOX using linoleic acid (Table 3). These results were similar to those of Clemente et al. (2000), who found that the LOX type BBL-1 produced more than 60% of 9-HPOD and *ca.* 40% of 13-HPOD. That study also purified and characterised the LOX isoenzymes from faba bean, named BBL-1 and BBL-2 (Clemente et al. 2000). The isoenzymes produced clearly different oxidation products from linoleic acid substrate. According to that study, BBL-1 produced both hydroperoxy- and keto-octadecadienoic acids, while only hydroperoxy-octadecadienoic acids were formed by BBL-2. It is important to know the proportions of hydroperoxides produced by faba bean LOX, because hydroperoxide isomers can break down through different pathways, resulting in different off-flavour products.

Faba bean LOX oxidised both linoleic and linolenic acids to produce various volatile lipid oxidation products, but trilinolein was a poor substrate and produced very little hexanal (Table 4). These results demonstrated that faba bean LOX preferred FFAs to acyl esters as substrates, which was found in another study of soybean LOX (Piazza and Nunez 1995). The profiles of volatile lipid oxidation products from linoleic and linolenic acids differed markedly (Table 4). For linoleic acid, hexanal was the dominating product, followed by 2-pentylfuran and 2-octenal. Hexanal may be produced by the faba bean LOX and HPL catalysed pathways, where HPL can scission 13-HPOD produced by LOX (Fauconnier and Marlier 1997; Gigot et al. 2010). In addition, the 1-hexanol may have been formed through ADH-catalysed lipid degradation from hexanal (Gigot et al. 2010). The 2-pentylfuran has been considered as a secondary lipid oxidation product originating from the scission of 9-HPOD (Belitz et al. 2009). However, for linolenic acid, 2,4-heptadienal (including 2,4-(*E*),(*Z*) and 2,4-(*E*),(*E*) stereoisomers) was the dominant product, followed by 2-hexenal, 2,4,6-nonatrienal and 3,5-octadien-2-one as the other main volatile products. These compounds were typical products of autoxidation of (n-3) PUFAs (Belitz et al. 2009).

Thus, LOX activity was found only in faba beans, and this activity could be affected by both the cultivars and the cultivation years, as well as the interactions between the two. Faba bean

LOX possessed the optimum pH value at 6, and the enzyme produced more 9-HPOD than 13-HPOD as primary products from linoleic acid. In addition, substrate was an important factor affecting the profile and amount of volatile products formed in faba bean. TAGs were not good substrates for LOX. Therefore, the potential to generate volatile compounds is higher when lipase activity is present in food matrices having lipids, which, together with LOX activity, lead to off-flavour problems.

6.2.3 High POX activity was found only in oat

In the present study (Study I), under incubation conditions developed and using cumene hydroperoxide as the oxygen donor, no epoxy products were produced from substrates with faba bean extract, showing that POX activity was either not present or quite low in faba bean samples. Nevertheless, hydroperoxide-dependent epoxidation in faba bean was proposed by Hamberg and Fahlstadius (1992). High POX activity was found in all oat samples, and earlier POX was isolated from oat grains (Hamberg and Hamberg 1996).

In this thesis, we investigated POX activity in oat cultivars from various cultivation years. Results showed that the activity was mainly affected by sample cultivars (Tables 5 and 6, Study I). Furthermore, the substrate specificity of oat POX was also determined by epoxidising various FFA and fatty acid methyl ester substrates. Oat POX converted both fatty acid methyl esters and FFA substrates to epoxy compounds. No products were found from TAGs, indicating that TAGs were unsuitable substrates for oat POX. Similarly, Meesapyodsuk and Qiu (2011) reported that oat POX used UFAs only with *cis*-double bonds and their methyl esters as substrates. The present research confirmed that oat POX preferred FFA substrates to their methyl esters to produce epoxy fatty acids (Tables 5 and 6, Study I). The overall amounts of epoxides formed from added fatty acid methyl esters ranged from *ca.* 0.25–0.55 mg (initial added substrates: 1.3 mg), whereas epoxides from FFAs ranged from *ca*. 0.5–1.1 mg (initial added substrates: 1.5 mg). In addition, oat POX catalysed linoleic acid (or methyl linoleate) to form two mono-epoxides in equal amounts, showing that epoxidation occurred similarly in both double bonds, as reported by Piazza et al. (2003b).

Although the amounts of epoxides formed from tri-unsaturated fatty acids and methyl esters were smaller than those formed from mono- and di-unsaturated ones, this does not necessarily mean that less epoxidation occurred with the tri-unsaturated substrates. This can be seen when looking at the measured amounts of substrate residues (Tables 5 and 6). With the tri-unsaturated substrates, the substrate residues were much smaller than with the other substrates, which means

that their products likely oxidised further during incubation and that the amount of epoxides decreased. Thus, when measuring POX activity, methyl oleate and oleic acid could be the substrates of choice, because they formed only one product and had a high proportion of epoxides.

In summary, no POX activity was found in faba bean, or else the activity was so low that it was undetectable using this method. Oat POX converted both FFAs and fatty acid methyl esters at various levels of unsaturation into mono-epoxy fatty acids, but FFAs were more preferred. In addition, oat POX did not distinguish the double bonds of linoleic acid. Finally, mono-unsaturated methyl oleate and oleic acid were shown to be good options for use as substrates when measuring POX activity.

6.3 Off-flavour related lipid oxidation products in oat, and epoxy and hydroxy fatty acids as the major non-volatile compounds (Study II)

6.3.1 NVOFAs occurred and were abundantly formed in NHT oat samples during storage

The present research first investigated the occurrence of NVOFAs in several old, stored oat samples (O-HT-F1, O-HT-F2 and O-NHT-P). Then, a preliminary study was done under uncontrolled experiment condition to observe the formation of NVOFAs in oat flours during storage (F-NHT-F1 and F-NHT-F2). Finally, a controlled experiment was conducted to investigate the effect of heat treatment on the formation of NVOFAs in oat samples during storage. Although POX activity has been found in oat (Piazza et al. 2003b; Piazza and Foglia 2005; Meesapyodsuk and Qiu 2011; Benaragama et al. 2017), the occurrence and formation of epoxy and hydroxy fatty acids in oat have been investigated in only one study focusing on oat grains and flours (Doehlert et al. 2010). However, that study analysed the epoxy and hydroxy fatty acid contents only before and after 22 weeks of storage; it did not monitor changes during storage. Epoxy and hydroxy fatty acids were observed by Leonova et al. (2008) in 33 oat varieties, but their study mentioned neither the process nor the storage history of the samples.

When the occurrence of NVOFAs was investigated using NHT and HT oat samples, the amounts differed in the NHT samples O-NHT-P, F-NHT-F1 and F-NHT-F2, which might reflect their different storage histories. All samples contained hydroxy fatty acids primarily oxidised from linoleic acid and epoxy fatty acids oxidised from oleic and linoleic acids (Table 7). Because no NVOFAs were found in HT oat samples (O-HT-F1 and O-HT-F2), it means that the NVOFAs were more likely formed through enzyme-catalysed lipid oxidation.

The formation of NVOFAs in NHT samples F-NHT-F1 and F-NHT-F2 was further studied during storage (at 35 ^{o}C for up to 24 weeks) to indicate POX-catalysed oxidation. Hydroxy and epoxy fatty acids (C3-C7, Table 7) were the dominant compounds among the total NVOFAs formed during storage of F-NHT-F1 sample. They increased markedly and dominated until week 18 of storage, after which they decreased. However, the formation of NVOFAs in F-NHT-F2 differed slightly: the hydroxy and epoxy fatty acids were the dominant compounds until week 12 of storage, after which they decreased and epoxy-hydroxy fatty acids increased remarkably (C1 and C2, Table 7). This might be due either to inactivation of the POX activity or lack of substrates as storage continued. Furthermore, some chemically unstable epoxide products may have decomposed and reacted further to become other compounds.

To further investigate the effect of storage on the formation of NVOFAs in well-characterised NHT and HT oat flours and the relations between NVOFA formation and other degradation reactions of lipids, a controlled storage experiment was conducted (at 40 ^{o}C for up to four months) using the oat samples NHT/HT-CV1, NHT/HT-CV2 and NHT/HT-IS. The results clearly showed that the NVOFAs were formed in all the NHT and HT oat samples but that the amounts differed markedly (Tables 8 and 9). Epoxy and hydroxy fatty acids already existed in some of the freshly milled NHT samples, including NHT-IS and NHT-CV2, indicating that the formation of the products may occur immediately after milling. In the NHT oat samples, the contents of NVOFAs reached their peak values during the first two months of storage, after which they declined markedly, especially by the end of the storage period (Table 8). Only minor levels of NVOFAs were produced in HT samples (Table 9), which is in line with our hypothesis that POX is inactivated when heated together with other enzymes. However, there are no published studies on the inactivation of POX.

The NHT oat samples contained more epoxy fatty acids than hydroxy fatty acids until they reached the peak total amounts of NVOFAs, after which the proportion of hydroxy fatty acids increased. The values of epoxy-octadecanoic acid (C7, Table 8) were usually higher than the sum of the epoxy-octadecenoic acids (C5+C6, Table 8), indicating that oleic acid was as efficient in accepting oxygen via POX-catalysed epoxidation as was linoleic acid. This can be demonstrated by the comparable amounts of epoxides produced from oleic and linoleic acid substrates (Table 6), and also it was known that the proportion of both oleic and linoleic acid from the total fatty acids in oat is about 30–40% (Zhou et al. 1999). In addition, some other

minor lipid oxidation products, including 12,13-dihydroxy-octadec-9-enoic acid (C1), 13-hydroxy-9,10-epoxy-octadec-11-enoic acid and 9-hydroxy-12,13-epoxy-octadec-10-enoic acid (C2), were formed due to the further reactions of epoxy and hydroxy fatty acids. They occurred only after four months of storage in the NHT-CV1 sample and in small amounts formed during storage in the NHT-CV2 sample (Table 8). A study stored NHT and HT oat grains and their corresponding flour samples at 65% relative humidity and 37 °C for up to 22 weeks (Doehlert et al. 2010). The results showed that the flour from NHT oat grains was more prone to accumulate epoxy and hydroxy fatty acids than were the NHT grains. After storage, epoxides and hydroxides accounted for 5.0% and 1.1% of the total fatty acids in flour and 1.9% and 0.9% in the grains, respectively. The present research showed that marked changes in NVOFAs occurred during different storage durations: first the amounts increased, but then they decreased. In addition, Doehlert et al. (2010) found that steam treatment inhibited 60% and 17% of the formation of epoxy and hydroxy fatty acids, respectively; however, the present research found that heat treatment prevented fatty acid formation much more efficiently.

In summary, NVOFAs formed abundantly in the flours from NHT oat grains, with epoxy and hydroxy fatty acids as the major compounds. However, the epoxy fatty acids may decompose and disappear with prolonged storage. Other compounds, including epoxy-hydroxy and di-hydroxy fatty acids, occurred only after long-term storage. In addition, the amounts of NVOFAs formed in the HT oat samples were very small, confirming the importance of heat treatment in inactivating POX activity during oat processing.

6.3.2 Formation of NVOFAs was related to release of FFAs and volatile compounds in flours from NHT and HT oat grains

Because oat POX was shown not to oxidise unhydrolysed TAGs (Study I), the formation of FFAs was studied during the storage of flours from NHT and HT oat grains to investigate their relationship to the formation of NVOFAs. The initial low amounts of FFAs in freshly milled NHT oat samples (Fig. 18a) indicated that the lipid-hydrolysis process had begun immediately during the milling process. Only small amounts of FFAs were released in HT oat during storage, confirming that the lipase was inactivated, while large amounts of FFAs were found after the storage of NHT oat samples for one month (Fig. 18a), during which NVOFAs and volatile products also formed abundantly. Thus, the release of FFAs was positively correlated with the formation of NVOFAs. Of the three NHT oat samples, NHT-CV1 contained a smaller amount of FFAs and a larger amount of TAGs than the other two samples (Fig. 18), resulting in slower

formation of NVOFAs. It is worth noting that only after four months of storage did the TAGs in NHT-CV1 degrade to a low level. In two other NHT samples, this occurred earlier in storage (Fig. 18b).

As the contents of the major volatiles hexanal and 2-pentylfuran increased, the lipid oxidation progressed intensively in the NHT oat samples (Fig. 19). These two compounds were also reported as the major volatile lipid oxidation products in other studies on oat (Molteberg et al. 1996; Lampi et al. 2015), while their presence in the HT oat flours was very small. The formation of hexanal and 2-pentylfuran showed that the lipids oxidised more in NHT-IS than in other samples. In NHT-IS, their values reached their peaks after two months of storage, after which they decreased. In this sample, the peak value of the volatiles occurred at the same time that the TAGs were completely hydrolysed, and at the same time, the NVOFAs reached their peak values, after which they began to decrease. Although lipids in the NHT-CV2 sample were hydrolysed faster than in the NHT-CV1 sample, the formation of hexanal, which indicated lipid oxidation, was the opposite. In contrast, the formation of 2-pentylfuran, which is considered an indicator of enzymatic lipid oxidation (Ho and Chen 1994), was slightly higher in the NHT-CV2 sample than in the NHT-CV1 sample. This might have been due to higher enzyme activity in the NHT-CV2 sample. In line with this, the NVOFAs were already present in the NHT-CV2 sample before storage, and the formation of dihydroxy octadecenoic acid and hydroxy-epoxy-octadecenoic acids and the total amount of NVOFAs usually remained higher in the NHT-CV2 sample than in the NHT-CV1 sample. Each NHT oat sample produced much more 2-pentylfuran than hexanal, indicating that enzymatic lipid oxidation was important. Furthermore, the low formation of hexanal and 2-pentylfuran in the flours from HT oat grains showed that the inhibition of enzymes by heat treatment remarkably retarded lipid oxidation.

In summary, the importance of the role of lipid-modifying enzymes in oat was supported by the fact that the formation of NVOFAs was in line with the release of FFAs by lipase. Although LOX activity was not found in the present research, earlier studies reported oat had low LOX activity (Lehtinen and Kaukovirta-Norja 2011). Thereafter, hydroperoxides were produced either by autoxidation or by LOX-catalysed lipid oxidation. Higher amounts of NVOFAs and volatile products were formed in the flours from NHT oat grains than in HT oat. The high contents of these off-flavour compounds showed the importance of enzymatic lipid oxidation in stored oat. Finally, the process of lipid degradation was significantly retarded by the heat treatment process.

6.4 Faba bean lipid-modifying enzymes promoted formation of volatile lipid oxidation products in food models

Faba bean extract and RO and ROFA emulsions containing faba bean extract were used as food models to study the role of faba bean lipid-modifying enzymes in promoting the formation of volatile lipid oxidation products (Tables 10 and 11). Faba bean contains about 1.4% lipids, and linoleic acid and linolenic acid account for *ca.* 54% and 5% of total fatty acids, respectively (Lizarazo et al. 2015). In faba bean extract, only the endogenous lipids were present to react with the faba bean enzymes. In the emulsion with 3% and 5% of added RO/ROFA, mainly the TAGs in the RO and the FFAs in the ROFA were expected to begin reacting during the emulsification step. For the RO emulsion, hydrolysis by lipase was needed to enable the LOX reaction, and for the ROFA emulsion, no hydrolysis was needed.

In the faba bean extracts, hexanal was the main product, followed by 2-pentylfuran, 1-hexanol and 1-octen-3-ol. Hexanal accounted for *ca.* 75% of the total volatile compounds at pH 5, but at pH 6.4, its value decreased and the contents of other volatiles increased (Table 10). At this pH, the lipid oxidation was enhanced by the high level of LOX activity and by a variety of other enzymes (Fauconnier and Marlier 1997; Grechkin 2002) that enhanced the formation of other volatile products. However, at pH 8, lipid oxidation and the formation of volatiles were much less than at pHs 5 and 6.4, which is in line with the low level of LOX activity at pH 8. The present research found that faba bean LOX possessed activity at pHs 5 and 6.4 (Fig. 17), while faba bean lipase had high activity at pHs 7.5 and 8 (Fig. 15). The volatile compounds identified in this research were also reported by Oomah et al. (2014) as occurring in Canadian-grown faba bean, which shows that the compounds measured in this research were typical volatile products of faba bean. In addition, some of the major typical volatiles detected in the present research, such as hexanal, heptanal, 2-pentylfuran and octanal, were identified by Akkad et al. (2019) from faba bean samples having low or high tannin contents.

When RO was included in the emulsions made with faba bean extracts, the contents of volatiles increased remarkably (Table 10). The pH 6.4 was considered an ideal condition for several relevant enzymes. Because of the short reaction time, at this pH, the volatile compounds were mainly formed by enzyme catalysed lipid degradation and their formation was greater than at the other two pHs, indicating that both faba bean lipase and LOX possessed activity, although faba bean lipase prefers alkaline pH reaction conditions (Fig. 15a). RO exerted great influence

on the profiles of the compounds formed, because 2,4-heptadienal and (*E*)-2-pentenal, which are considered characteristic products of linolenic acid (Gardner 2003), were measured only in these emulsions. At pH 5, the formation of volatile products was not great (Table 10), because lipase activity was low and TAGs were not good substrates for faba bean LOX (Table 4). However, the formation of volatile lipid oxidation products at reaction pH 8 was the smallest among the three pH reaction conditions, mainly because faba bean LOX activity was markedly inhibited at alkaline pH values (Fig. 17) and thus, hydroperoxides were hardly formed. On the other hand, volatile products may form abundantly in the emulsion made from RO and faba bean extract. Gürbüz et al. (2018) studied the formation of volatile products in oil-in-water emulsions made from purified RO (with tocopherols removed) and faba bean water extracts. They found that the volatile compounds, including (*E*)-2-pentenal, hexanal, (*E*)-2-hexenal, 2-heptanone, 2-pentylfuran, (*E*)-2-heptenal, octanal, (*E*,*E*)-2,4-heptadienal, 3-octen-2-one, nonanal and (*E*,*E*)-3,5-octadien-2-one, were the major products and formed markedly after seven days of storage. However, their study used purified RO that was more prone to autoxidation, especially when the oil emulsions containing faba bean extracts were stored.

Volatile lipid oxidation products formed more abundantly in ROFA emulsions than in RO emulsions, showing that FFAs were more prone to oxidation than TAGs and were oxidised by faba bean LOX to form hydroperoxides, resulting in the much-increased formation of volatiles (Tables 4 and 11). It is worth noting that in RO emulsions containing faba bean extract, the proportion of the total amounts of volatile lipid oxidation products accounted for by hexanal ranged from 34–69%, while the values measured in ROFA emulsions were only 19–32% (Tables 10 and 11). Thus, further reactions may become more obvious during more extended oxidation. It was interesting that octanoic acid was detected in all ROFA emulsions and in the RO emulsion at pH 6.4. This compound could be formed by the scission of 9-hydroperoxide from linoleic acid (Schaich et al. 2013). Another typical product was (*E*,*E*)-3,5-octadien-2-one, formed from linolenic acid, which occurred only in the ROFA emulsions.

In summary, volatile lipid oxidation products already existed in faba bean extract. It was clear that the amounts of these volatile products formed from faba bean extract and RO or ROFA emulsions with extract were influenced markedly by the reaction pHs. In general, the maximum amounts of the compounds were detected at pH 6.4 for faba bean extract. At each pH level, the overall volatile formation from ROFA emulsion was usually higher than that from RO emulsion or faba bean extract. Hexanal was always the dominating compound in both RO and ROFA

emulsions with different oil concentrations, but its formation ratio in ROFA emulsion was higher than that in RO emulsion (up to over 40%, Table 10 and Table 11).

6.5 Overall evaluation of lipid-modifying enzymes as causes of off-flavours in oat and faba bean

A scheme was created to evaluate the potential of the three studied lipid-modifying enzymes to form off-flavours in oat and faba bean (Fig. 20). The different activity levels of the lipid-modifying enzymes in oat and faba bean lead to the formation of non-volatile and volatile products through different pathways.

This thesis showed that high levels of lipase and POX activities occurred in oat samples, while no LOX activity was found. Oat lipase can hydrolyse TAGs rapidly to release FFAs in the flours from NHT oat grains. Due to the low LOX activity in oat, the UFAs are mainly subjected to autoxidation and then yield the hydroperoxides for further reactions. But the autoxidation process is slow. POX is responsible for the formation of hydroxy and epoxy-hydroxy fatty acids, which were suggested to cause undesirable bitterness in foods (Baur et al. 1977; Biermann et al. 1980). NVOFAs formed abundantly during the long-term storage of NHT oat flours. The much slower formation of hydroperoxides by autoxidation than by LOX-catalysed lipid oxidation in NHT oat flours explains why the formation of neither volatile nor non-volatile oxidation products was rapid in HT samples but they formed gradually over prolonged storage. Heat treatment has long been used as an efficient food-processing method to inactivate lipid-modifying enzyme activities, controlling both the formation of off-flavour compounds and the release of FFAs, as seen in oat flours from HT oat grains. Overall, high lipid contents, together with endogenous lipid-modifying enzymes, enhance the risks of lipid-derived off-flavour problems in oat products.

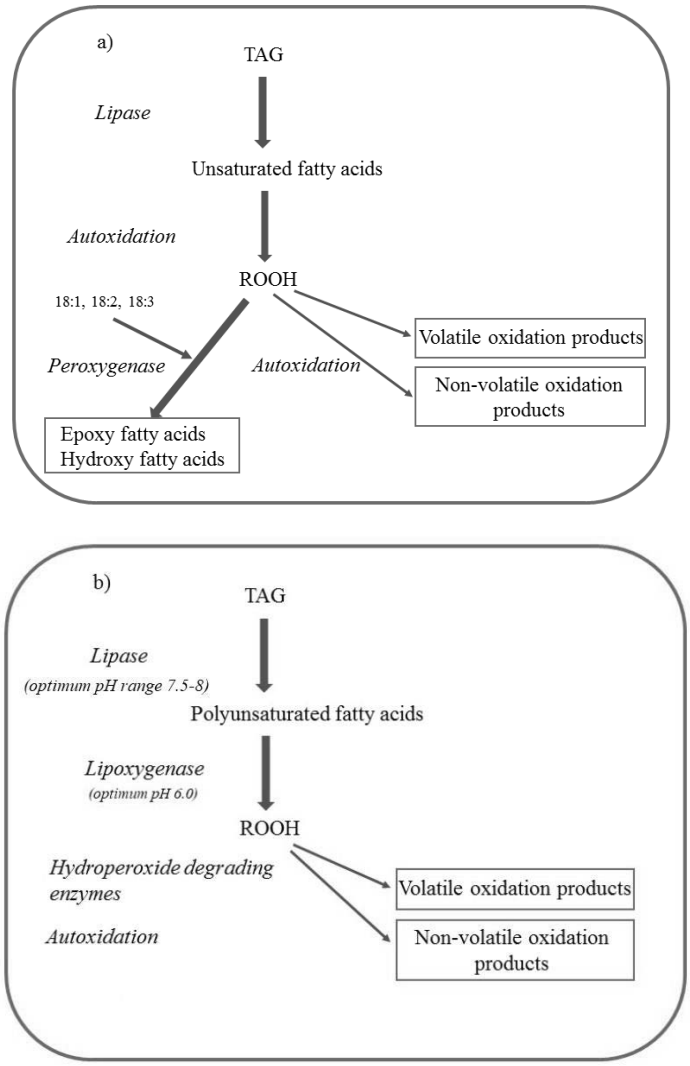


Fig. 20. Possible schemes by which enzymatic and chemical reactions cause lipid-derived off-flavours in a) oat and b) faba bean.

High levels of lipase and LOX activities were measured in faba bean samples, but no POX activity was found, which shows that the epoxy and hydroxy fatty acids are less important off-flavour compounds in faba bean than they are in oat. The high levels of lipase and LOX activity occurring in faba bean may lead to the rapid formation of hydroperoxides. However, the markedly differing pH optima of faba bean lipase and LOX may influence this reaction pathway. Faba bean lipase possesses high activity at pH 7.5–8, while the LOX optimum pH was pH 6. Faba bean lipase catalysed the hydrolysis of triolein, trilinolein and RO TAGs to release FFAs.

Its activity is needed for reactions of the LOX pathway, because LOX prefers to oxidise PUFAs more than TAGs to form hydroperoxides. In addition, hydroperoxide-degrading enzymes, such as HPL and ADH, contributed to the formation of volatile off-flavour compounds. The profiles of volatile products formed by faba bean LOX pathway and other relevant enzymes highly depended on the substrates. This thesis confirmed that when faba bean is used as a food ingredient, the formation of volatile lipid oxidation products occurs rapidly in the RO and ROFA emulsions containing faba bean extract, and that the formation of the compounds is affected by the pH of the food systems. Thus, the risk of high levels of lipase and LOX activity in faba bean was well evaluated, and they were proven to be important potential enzymes responsible for the 'beany flavour' of faba bean.

Even though there were differences between the lipid-modifying enzyme activities in oat and faba bean among cultivars and cultivation years, these differences were modest and might not influence the formation of off-flavours. Furthermore, these enzyme activities present in oat and faba bean should be controlled when using oat and faba bean as ingredients for foods rich in lipids. Thus, in both seeds, it is crucial to control the lipid-modifying enzymes to enable stable and high-quality food products.

7 CONCLUSIONS

The increasing use of plant-derived foods, especially their proteins, is a global trend. One of the challenges of using cereal grains and legumes as sources of protein is the undesirable flavours associated with them. Many undesirable off-flavour compounds are formed from lipids as a result of complex enzymatic and chemical reactions. Thus, it is essential to understand and control lipid-modifying enzymes in order to prolong the shelf life of cereal and legume ingredients and products. The focus of this thesis was to investigate the activities of the lipid-modifying enzymes, lipase, LOX and POX, and to study the formation of their reaction products from lipids in selected oat and faba bean materials.

To measure POX activity, a method was developed based on the specific production of epoxy compounds from substrates and using cumene hydroperoxide as an oxidant in a reaction carried out for 1 h at pH 7 at room temperature. The epoxy products were measured using the GC-FID/MS and UHPLC-ELSD/MS methods, which were shown to be reliable and sensitive. The comparison of fatty acid methyl esters and FFAs at various levels of unsaturation and TAGs as substrates showed that POX was more active toward FFAs than toward their methyl esters in producing epoxy compounds and that no epoxides were formed from TAGs. The knowledge of substrate specificity obtained in the present research supports the importance of the substrate chosen to the reliable measurement of POX activity. Mono-unsaturated methyl oleate and oleic acid are suggested for use in measuring POX activity in oat-based foods, because they formed only one epoxide and the product remained structurally stable. To study the formation of NVOFAs in oat, the ASE and SPE methods that were developed enabled extraction and purification of NVOFAs from oat samples with good recovery of the added epoxy and hydroxy fatty acids, showing that the extraction was efficient and did not degrade the NVOFAs. The products were further reliably analysed by UHPLC-ELSD/MS. This thesis provides a reliable approach to study further of POX activity in food materials. In addition, the UHPLC-ELSD/MS method can also measure the NVOFAs occurring in food matrices, which is helpful in indicating the level of lipid oxidation and in studying the role of NVOFAs as off-flavour compounds in plant-derived foods.

High levels of lipase and POX activity occur in oat. The lipase activity may be affected by both the oat cultivars and cultivation years as well as the interactions between the two, but the POX activity was affected only by cultivars. Oat lipase will begin to hydrolyse the TAGs as soon as the oat grains are broken down. The majority of FFAs released in oat are unsaturated ones,

which are prone to autoxidation and LOX-catalysed lipid oxidation into hydroperoxides. Although no LOX activity was found in oat, autoxidation can occur gradually during long-term storage. The high level of POX activity occurring in oat is responsible for the formation of NVOFAs in NHT oat during storage, but their formation is hindered by the heat treatment used in industrial milling. Epoxy and hydroxy fatty acids were the primary NVOFAs that formed during storage, while other compounds, notably dihydroxy and epoxy-hydroxy fatty acids, began to be formed only after extensive oxidation and remained at low levels. Changes in the NVOFA profile together with changes in their total content may result in differences in off-flavours developed during storage. This research found that several lipid-modifying enzymes catalysed the reactions during the storage experiment of oat. The reason for this was that the production of NVOFAs was in line with the release of FFAs by lipase and with the formation of hydroperoxides by either autoxidation or LOX. Finally, heat treatment is an effective approach to inactivating lipase and POX activity in oat.

Faba bean possesses high levels of lipase and LOX activity. However, POX activity was not found. The lipase activity was mainly affected by the cultivars, while the LOX activity was affected by both the cultivars and the cultivation years as well as by the interactions between the two. Faba bean lipase had activity under neutral and alkaline pH conditions. The optimum pH of lipase was found to be pH 8 using *p*-NPB as a substrate. However, lipase catalysed more hydrolysis of TAGs at pH 7.5 than at pH 8, in the order of triolein > trilinolein > RO TAGs. The optimum pH of faba bean LOX was found to be pH 6. Faba bean LOX produced more 9-HPOD than 13-HPOD from linoleic acid. Together with some other lipid-degrading enzymes, such as HPL and ADH, linoleic and linolenic acids formed large amounts of typical volatile compounds. However, only a small amount of hexanal was formed from trilinolein. Furthermore, the food-model study, which included the faba bean extract and the RO and ROFA emulsions containing extract, showed that faba bean lipase and LOX are capable of catalysing the degradation of faba bean lipids as well as added lipids. Adding RO to form emulsions promoted the formation of volatile lipid oxidation products, and adding ROFA increased this formation even more. In both food models, the formation of volatile profiles was markedly affected by the reaction pH. However, more research is needed on the effects of lipase on the formation of off-flavour compounds during long-term storage. The inactivation of lipase and LOX activities is crucial to maintaining high-quality of faba bean foods.

This thesis studied the potential of the reactions catalysed by lipid-modifying enzymes and chemical lipid oxidation reactions to form lipid-derived off-flavour compounds in oat and faba bean. The approach developed to measure POX activity and the UHPLC-ELSD/MS method developed to analyse NVOFAs in oat and other matrices are reliable. NVOFAs are formed rapidly during storage of NHT oat, but the heat treatment used to inactivate lipase in the food industry was shown to also inactivate POX. The occurrence of lipase and LOX together in faba bean may promote the formation of volatile lipid oxidation products through the LOX pathway. Thus, comprehensive study of the lipid-modifying enzymes is needed to yield the knowledge necessary to develop stable, high-quality food products based on oat and faba bean.

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